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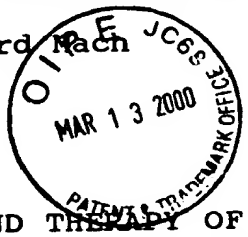

Applicants: Bernard Conrad and Bernard Mach

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For : METHODS FOR DIAGNOSIS AND THERAPY OF AUTOIMMUNE DISEASE, SUCH AS INSULIN DEPENDENT DIABETES MELLITUS, INVOLVING RETROVIRAL SUPERANTIGENS

1185 Avenue of the Americas
New York, New York 10036
March 10, 2000

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

TRANSMITTAL OF CERTIFIED COPIES OF EARLIER FILED FOREIGN APPLICATIONS AND CLAIM TO PRIORITY PURSUANT TO 35 U.S.C. §119

Applicants submit herewith as Exhibit A, a certified copy of European Patent Application No. 97112482.1, filed on July 22, 1997, and as Exhibit B, a certified copy of European Patent Application No. 97401773.3, filed on July 23, 1997. Both of which are cited in applicant's Declaration pursuant to 37 C.F.R. §1.63.

Applicant hereby claims the benefit of the July 22, 1997 filing date pursuant to 35 U.S.C. §119 and 37 C.F.R. §1.55(a).

Respectfully submitted,

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Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

97112482.1

Der Präsident des Europäischen Patentamts:
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Methods for Diagnosis and Therapy of Autoimmune Disease, such as Insulin Dependent Diabetes Mellitus, involving Retroviral Superantigens.

The present invention relates to methods for the diagnosis of human autoimmune disease, for example Insulin Dependent Diabetes Mellitus (IDDM), and to methods for identifying substances which can be used in the therapy and prevention of such diseases. The invention further relates to novel human retroviruses involved in autoimmune disease and having superantigen activity, as well as to their expression products.

For some autoimmune diseases such as IDDM, Multiple Sclerosis, arthritis and others, it is known that a combination of genetic, environmental and possibly exogenous infectious factors may be important in precipitating disease. However, the precise roles of each of these factors remains incompletely elucidated. For example, for IDDM, the Major Histocompatibility Complex (MHC) Class II genotype is one of the strongest genetic factors determining disease susceptibility (Vyse, T.J.and Todd J.A., 1996) although the respective roles of the different MHC Class II^+ cell types in promoting disease has not yet been clarified. Furthermore, IDDM shows temporal, epidemic-like variations and the clinical disease exhibits preferential seasonal onset (Karvonen et al., 1993). Recently, Conrad et al. (1994) provided evidence for superantigen involvement in IDDM aetiology and postulated that viruses may be the modifying agent responsible for the presence of superantigen on diabetic islets.

Genetic background also has an important influence in multiple sclerosis. In addition, Perron et

al (Perron et al, 1997) have recently identified a retrovirus which can be isolated from cells of multiple sclerosis patients. Whether the retrovirus contributes as a causative agent of multiple sclerosis or as a link in the pathogenic process, or whether it is merely an epiphenomenon, has not been identified. No superantigen activity of the retrovirus has been identified.

It is an aim of the present invention to identify agents implicated in the pathogenesis of human autoimmune diseases, such as IDDM, and on the basis of these agents to provide reliable diagnostic procedures and therapeutic or prophylactic substances and compositions.

These objectives are met by the provision, according to the invention, of diagnostic procedures involving the detection of expressed retroviruses having superantigen (SAg) function, these retroviruses being directly involved in the pathogenesis of human autoimmune disease by activation of autoreactive T-cells. Compounds and compositions capable of blocking SAg function or production are also provided as therapeutic and prophylactic agents in the treatment of autoimmune disease.

The present invention is based on the discovery, by the present inventors that superantigens (SAgs) encoded by retroviruses, particularly endogenous retroviruses, play a major role in the pathogenesis of autoimmune disease, very likely by activating autoreactive T-cells.

Superantigens (SAgs) (Choi et al, 1989 ; White et al, 1989) are microbial proteins able to mediate interactions between MHC Class II^+ - and polyclonal T-cells resulting in reciprocal activation (Acha-Orbea et al, 1991 ; Choi et al, 1991 ; Fleischer and Schrezenmeier, 1988). Their function is restricted by only two absolute requirements : the presence of MHC

Class II on the surface of the presenting cells and the expression of one or more defined Variable (V)-β T cell receptor (TCR) chain(s) on T cells.

The potential role of SAgs in human diseases is ill-defined. Bacterial SAgs have been proposed to be associated with the pathogenesis of autoimmune disease (White et al, 1989). However, although pathogen disease associations have been described, none of these have as yet implicated a pathogen-encoded SAg (Howell et al, 1991 ; Paliard et al, 1991). A SAg-like activity resembling the one encoded by MMTV has been reported to be associated with herpesvirus infections (Dobrescu et al, 1995 ; Sutkowski et al, 1996). However, in none of these two systems has it been demonstrated that the SAg activity is actually encoded by the infectious agent. SAg activity has been reported in patients having Type I diabetes (Conrad et al 1994). However, the origin of the Sag activity is not identified.

In the framework of the present invention, the inventors have identified the source of SAg activity in IDDM patients as being a novel endogenous retrovirus, (HERV) designated $IDDKK_{1.2}$-22. This retrovirus is related to, but distinct from mouse mammary tumor virus (MMTV). It is ubiquitous in the human genome but is only expressed in diabetic individuals, possibly in response to a particular environmental stimulus. The HERV encodes superantigen (SAg) activity within the <u>env</u> gene. Expression of the SAg gives rise to preferential expansion of Vβ-7 T-cell receptor positive T-cells, some of which are very likely to be autoreactive. Thus the expression of self-SAg leads to systemic activation of a sub-set of T-lymphocytes, among which autoreactive T-cells, will in turn give rise to organ-specific autoimmune disease.

The involvement of retroviral SAg, particularly endogenous retroviral SAg in autoimmune disease is

unexpected. Indeed, endogenous retroviruses (HERV) form an integral part of the human genome. If expressed from birth, any autoreactive T-cells activated by expression of a retroviral SAg should be deleted as part of the normal development of the immune system (thymic deletion). However, in the case of autoimmune diseases such as diabetes, the expression of the retrovirus, and hence of the encoded SAg, occurs only later in life, leading to the proliferation of autoreactive T-cells.

To identify the microbial agent responsible for SAg activity in diabetes, the present inventors have developed a novel primer-extension technique. This method can be used to isolate and identify, in a sample of polyadenylated RNA, any expressed, previously unidentified retroviral RNA, particularly retroviruses having SAg activity and being involved in human autoimmune disease. The method comprises the following steps :

i) isolation of the 5' R-U5 ends of expressed putative retroviral genomes using nucleic acid amplification, the 3' primer being complementary to known « primer binding sites » (pbs).

ii) isolation of the 3' R-poly(A) ends corresponding to the 5' R-U5 ends, by use of primers specific for the R regions isolated in step i).

iii) amplification of the conserved RT-RNase H region within the pol gene by using degenerate primers corresponding to the conserved region.

iv) amplification of the 5' moiety of the putative retroviral genome by using primers specific for the different U5 regions isolated in step i) in conjunction with a primer specific for the 3' end of the central pol region isolated in step iii).

v) amplification of the 3' moiety of the putative retroviral genome using primers specific for the central pol region isolated in step iii) in conjunction

with primers specific for the poly(A) signals present in the 3' R-poly(A) sequences isolated in step ii).

vi) confirmation of the presence of an intact retroviral genome by amplification using primers specific for its predicted U5 and U3 regions.

Once an expressed retrovirus has been identified, its SAg activity can be tested by contacting a biological sample containing MHC class II^+ cells expressing the putative Sag activity, with cells bearing one or more variable (V)-β T-cell receptor (TCR) chains and detecting preferential proliferation of a Vβ subset.

The techniques developed by the inventors to elucidate Sag involvement in IDDM, can be used to identify the possible involvement of expressed retrovirus and encoded SAg activity in other autoimmune diseases. The characterisation of the retrovirus and its SAg can then be made, and the particular Vβ-T cell receptor chain activation associated with the SAg can be identified. A given autoimmune disease can thus be fully characterised by reference to a retroviral Sag specifically associated with the disease, and to the Vβ-specificity. Once these criteria have been determined, specific diagnostic, therapeutic and prophylactic tools can be elaborated for each autoimmune disease involving retroviral SAg-stimulation of autoreactive T-cells.

The present invention involves, in a first embodiment, methods of diagnosis of autoimmune disease based on the specific expression, in autoimmune patients, of retroviruses having Sag activity.

The methods of diagnosis of the present invention are advantageous in so far as they are highly specific, distinguishing between expressed and non-expressed viral nucleic acid, and can thus be reliably used even

when the pathological agent is a ubiquitous endogenous retrovirus. They can be carried out on easily accessible biological samples, such as blood or plasma, without extensive pre-treatment. The diagnostic methods of the invention detect disease-specific expression of the retrovirus and can thus be applied before appearance of clinical symptoms, for example on genetically predisposed individuals. This allows suitable therapy to be initiated before autoimmune destruction of a particular target tissue occurs.

In the context of the present invention, the following terms encompass the following meanings :

- a « human autoimmune disease » is defined as a polygenic disease characterised by the selective destruction of defined tissues mediated by the immune system. Epidemiological and genetic evidence also suggests the involvement of environmental factors.
- a « human endogenous retrovirus » (HERV) is a retrovirus which is present in the form of proviral DNA integrated into the genome of all normal cells and is transmitted by Mendelian inhertance patterns. Such proviruses are products of rare infection and integration events of the retrovirus under consideration into germ cells of the ancestors of the host. Most endogenous retroviruses are transcriptionally silent or defective, but may be activated under certain conditions. Expression of the HERV may range from transcription of selected viral genes to production of complete viral particles, which may be infectious or non-infectious. Thus, in some cases, endogenous retroviruses may also be present as exogenous retroviruses.
- a Superantigen is a substance, normally a protein, of microbial origin that binds to major histocompatibility complex (MHC) Class II molecules

and stimulates T-cell, via interaction with the Vβ domain of the T-cell receptor (TCR). SAgs have the particular characteristic of being able to interact with a large proportion of the T-cell repertoire, i.e. all the members of a given Vβ subset or « family », or even with more than one Vβ subset, rather than with a single, defined member of a Vβ family as is the case with a conventional (MHC-restricted) antigen. The superantigen is said to have a mitogenic effect that is MHC Class II dependent but MHC-unrestricted. SAgs require cells that express MHC Class II for stimulation of T-cells to occur.

- « SAg activity » signifies a capacity to stimulate T-cells in an MHC-dependent but MHC-unrestricted manner.
- a retrovirus having SAg activity is said to be « associated with » a given autoimmune disease when expressed retroviral RNA can be found specifically in biological samples of autoimmune patients. Preferably « associated with » further signifies in this context that retroviral SAg activation of a Vβ subset gives rise directly or indirectly to proliferation of autoreactive T-cells targeting tissue characteristic of the autoimmune disease. Blockage of SAg activity thus normally prevents generation of autoreactive T-cells.

More particularly, in a first embodiment, the present invention relates to a process for the diagnosis of a human autoimmune disease, including pre-symptomatic diagnosis, said human autoimmune disease being associated with human retrovirus having Superantigen (SAg) activity, comprising specifically detecting in a biological sample of human origin at least one of the following :

I : the mRNA of an expressed human retrovirus known to have Superantigen (SAg) activity, or fragments of such expressed retroviral mRNA, said retrovirus being associated with a given autoimmune disease, or

II : protein expressed by said retrovirus, or

III : antibodies specific to the proteins expressed by said retrovirus, or

IV : SAg activity specifically associated with the autoimmune disease.

Thus, the diagnosis of a given autoimmune disease can be made, according to the invention, by one or more of four methods (I to IV), each involving the detection of a specific aspect of a SAg-encoding retrovirus associated with the autoimmune disease, particularly an endogenous retrovirus.

Each of the four possible methods I to IV of diagnosis of human autoimmune disease will be described in detail below.

According to method I, the autoimmune disease is diagnosed by specifically detecting in a biological sample the mRNA of an expressed human retrovirus known to have SAg activity.

Specific detection of retroviral expressed mRNA is preferably carried out using nucleic acid amplification with viral specific primers which discriminate between proviral DNA and expressed RNA template. This is of particular importance when the retrovirus associated with the autoimmune disease is an endogenous retrovirus. Indeed in such cases, the proviral DNA is present in all human cells, whether or not the autoimmune disease is present, thus leading to false positives.

The biological sample may be any body fluid or tissue but is preferably plasma or blood. Normally, total RNA is extracted from the sample using

conventional techniques. DNAse treatment may be carried out to reduce contaminating cellular DNA.

By performing the amplification on total RNA samples, the effects of contaminating DNA are reduced but not eliminated, even after treatment by DNAse. The method of the present invention allows selective amplification of expressed viral RNA transcripts using at least one m-RNA specific primer, for example a poly-A specific primer, even in the presence of contaminating viral DNA in the sample. The poly-A specific primer is specific for the poly-A signaals present in the R-poly(A) sequences and the 3' extremity of the retrovirus (see for example Figure 2A step 5 and Figure 2C).

It has surprisingly been found that a poly-A-specific primer having four T's is optimal for the purposes of the present invention, although five or more Ts may be included in the primer.

The mRNA specific amplification requires a reverse transcriptase (RT) step, for which the poly A-specific primer is also be used.

The second primer in the PCR step is generally complementary to the U3 region. When the amplification product has a size of about 300 to 500 nucleotides, the conditions applied for the amplification (PCR) step are normally the following :

i) reverse transcriptase	:	50°C	30 minutes
ii) amplification	:	94°C	2 minutes
(for a total		94°C	30 secondes
of 10 cycles)		68°C	30 secondes
		- 1.3°C	each cycle
		68°C	45 secondes

iii) amplification	:	94°C	30 secondes
(for a total		55°C	30 secondes
25 cycles)		68°C	45 secondes

The amplified material is subjected to gel electrophoresis and hybridised with suitable probes, for example generated from the U3 region.

By performing the mRNA specific detection of the invention, the presence of a given expressed retrovirus can be reliably determined in a biological sample. For endogenous retroviruses expression generally indicates onset of the disease process. This can be detected well before the apparition of any clinical symptoms. The diagnosis of the invention can thus be used to detect onset of the disease process, enabling treatment to be administered before irreversible autoimmune attack occurs.

The invention also encompasses pro-viral specific detection of retroviral DNA, and simultaneous detection of both expressed retroviral m-RNA and proviral DNA. Details of these methods are given in Figure 2D and 2E, and associated legends. Specific proviral DNA detection can be used on healthy biological samples to confirm the endogenous nature of the retrovirus. the assay detecting both retroviral mRNA and proviral DNA can be used as an internal standard.

According to a preferred embodiment of the invention, the autoimmune disease detected is IDDM. The present inventors have identified, a human endogenous retrovirus associated with IDDM. This novel retrovirus (called $IDDMK_{1.2-22}$) has SAg activity encoded in the NH_2 terminal portion of the *env* gene, causing preferential proliferation of Vβ7 - TCR chain bearing T-cells.

IDDMK$_{1.2}$-22 comprises the 5' LTR, 3' LTR and env-encoding sequences shown in Figures 7A, 7B and 7C respectively, and further comprises gag-encoding sequences. The SAg portion of the env protein occurs within the sequences shown in Figure 7D or 7G, particularly 7G.

Diagnosis of IDDM by specific detection of expressed retroviral RNA is carried out using a polyA specific probe of the type :

5' TTTTTGAGTCCCCTTAGTATTTATT 3'

or similar sequence specifically hybridising to the polyA region of IDDMK$_{1.2}$-22 type retroviruses, having at least 90% sequence identity with the IDDMK$_{1.2}$-22 and having SAg activity.

According to a second embodiment (II) of the invention, the human autoimmune disease associated with a retroviral SAg is diagnosed by specifically detecting protein expressed by the retrovirus, particularly gag, pol or env. In the case of endogenous retroviruses, the expressed proteins may be slightly different from the expected products as a result of read-through phenomena and possibly reading-frame shifts. Preferably, the expressed protein is detected in the biological sample, such as blood or plasma, using antibodies, particularly monoclonal antibodies, specific for the said protein. A Western-like procedure is particularly preferred, but other antibody-based recognition assays may be used.

In the case of IDDM, a preferred diagnostic method comprises the detection of a protein encoded by the env gene, as shown in Figure 7C, 7D or 7G, or the pol protein shown in Figure 7H, or the IDDMK$_{1.2}$-22 GAG protein. Alternatively, proteins having at least approximately 90 % homology with these proteins, or proteins arising from read-through of internal stop

codons, possibly with frame-shift, particularly a -1 frame shift, occurring immediately after the internal stop codon. Fragments of any of these proteins having at least 6, and preferably at least 10 amino acids may also be detected. Preferred proteins for this type of diagnostic assay are those having SAg activity. It is also possible to detect retroviral particles when produced.

According to a third embodiment (III) of the invention, the autoimmune disease is diagnosed by detecting in a biological sample, antibodies specific for the protein expressed by the associated retrovirus.

Detection of antibodies specific for these proteins is normally carried out by use of the corresponding retroviral protein or fragments thereof having at least 6 amino-acids. The proteins are typically Gag, Pol or Env or fragments thereof and may or may not have superantigen activity. The retroviral proteins used in the detection of the specific antibodies may be recombinant proteins obtained by introducing viral DNA encoding the appropriate part of the retrovirus into eukaryotic cell and the conditions allowing the DNA to be expressed and recovering the said protein.

In the context of the present invention, the terms "antibodies specific for retroviral proteins" signifies that the antibodies show no significant cross reaction with any other proteins likely to occur in the biological sample. Generally, such antibodies specifically bind to an epitope which occurs exclusively on the retroviral protein in question. The antibodies may recognize the retroviral protein having SAg activity as presented by the M.H.C class II molecule.

Detection of specific antibodies may be carried out using conventional techniques such as sandwich

assays, etc. Western blotting or other antibody-based recognition system may be used.

According to the fourth embodiment of the invention, the autoimmune disease is diagnosed by detecting in a biological sample SAg activity specifically associated with the autoimmune disease. This is done by contacting a biological fluid sample containing MHC class II+ cells with cells bearing one or more variable β-T-receptor chains and detecting preferential proliferation of the Vβ subset characteristic of said autoimmune disease.

The biological sample according to this variant of the invention is typically blood and necessarily contains MHC class II+ cells such as B-lymphocytes, monocytes or macrophages which have the capacity to bind the superantigen and enable it to elicit its superantigen activity. MHC class II content of the biological sample may be boosted by addition of agents such as IFN-gamma.

The biological fluid sample is contacted with cells bearing the Vβ-T receptors belonging to a variety of different families or subsets in order to detect which of the Vβ subsets is stimulated by the putative SAg, for example V-β2, 3, 7, 8, 9 13 and 17. Within any one V-β family it is advantageous to use V-β chains having junctional diversity in order to confirm superantigen activity rather than nominal antigen activity.

The cells bearing the V-β receptor chains may be either an unselected population of T-cells or T-cell hybridoma. If unselected T-cells are used, the diagnostic process is normally carried out in the following manner : the biological sample containing MHC Class II+ cells is contacted with the T-cells for

approximately 3 days. A growth factor such as Interleukin 2 (IL-2) which selectively amplifies activated T-cells is then added. Enrichment of a particular V-β family or families is measured using monoclonal antibodies against the TCR-β-chain. Only amplified cells are thus detected. The monoclonal antibodies are generally conjugated with a detectable marker such as a fluorochrome. The assay can be made T-cell specific by use of a second antibody, anti CD3, specifically recognizing the CD3-receptor.

T-cell hybridoma bearing defined T-cell receptor may also be used in the cell-based assay for SAg activity. According to this variant, activation of a particular family of V-β hybridoma leads to release of IL-2. IL2 release is therefore measured as read-out using conventional techniques. This procedure is illustrated in Figure 9.

For diabetes, detection of SAg activity will normally lead to preferential proliferation of the V-β7 subset. For other autoimmune diseases, other V-β subsets may be proliferated.

According to another aspect of the present invention, there is provided human retroviruses having superantigen activity and being associated with human auto immune disease. Such retroviruses are obtainable from RNA prepared from a biological sample of human origin, by carrying out the following steps :

i) isolation of the 5' R-U5 ends of expressed putative retroviral genomes using nucleic acid amplification, the 3' primer being complementary to known « primer binding sites » (pbs) ;

ii) isolation of the 3' R-poly(A) ends corresponding to the 5' R-U5 ends, by use of primers specific for the R regions isolated in step i) ;

iii) amplification of the conserved RT-RNase H region within the pol gene by using degenerate primers corresponding to the conserved region ;

iv) amplification of the 5' moiety of the putative retroviral genome by using primers specific for the different U5 regions isolated in step i) in conjunction with a primer specific for the 3' end of the central pol region isolated in step iii) ;

v) amplification of the 3' moiety of the putative retroviral genome using primers specific for the central pol region isolated in step iii) in conjunction with primers specific for the poly(A) signals present in the 3' R-poly(A) sequences isolated in step ii) ;

vi) confirmation of the presence of an intact retroviral genome by amplification using primers specific for its predicted U5 and U3 regions.

A preferred human endogenous retrovirus of the invention is IDDMK 1,2 22 comprising each of the sequences illustrated in figures 7A, 7B, 7C or sequences having at least 90 % identity with these sequences. This retrovirus has a size of approximately of 8.5 kb, has SAg activity encoded within the Env region and gives rise to V-β7 specific proliferation.

The invention also relates to proviral DNA of a retrovirus having superantigen activity and being associated with an autoimmune disease. Such proviral DNA is naturally found integrated into the human genome. The proviral DNA may be obtained from a biological sample of human origin by :

i) obtaining retroviral RNA according to the method of claim 13, and further,

ii) generating a series of DNA probes from the retroviral RNA obtained in i);

iii) hybridising under stringent conditions, the probes on a genomic human DNA library ;

iv) isolation of the genomic sequences hybridising with the probes.

The invention also relates to nucleic acid molecules comprising fragments of the retroviral RNA or DNA described above. The fragments may be either specific for a given retrovirus, specific signifying a homology of less than 20 % with other human or non-human retroviruses. Preferred nucleic acid molecules of the invention encode SAg activity particularly SAg activity responsible for the proliferation of autoreactive T-cells. If the region of the viral genome encoding the SAg activity is unknown, the particular region may be identified by :

i) transfecting expressed retroviral DNA or portions thereof into MHC Class II^+ antigen presenting cells under conditions in which the viral DNA is expressed,

ii) contacting the MHC class II^+ transfectants with cells bearing one or more defined (V)-β T-cell receptor chains, and

iii) determining whether the transfectant is capable of inducing preferential proliferation of a Vβ subset, the capacity to induce preferential proliferation being indicative of SAg activity within the transfected DNA or portion thereof.

The nucleic acid molecule encoding SAg activity may be derived from an endogenous human retovirus and may contain at least one internal stop codon.

The nucleic acid molecules of the invention may comprise ribozymes or antisense molecules to the retrovirus involved in autoimmune disease.

The invention also relates to nucleic acid molecules capable of hybridizing in stringent conditions with retroviral DNA or RNA. Typical stringent conditions are those where the combination of temperature and salt concentration chosen to be

approximately 12-20°C below the Tm (melting temperature) of the hybrid under study.

Such nucleic acid molecules may be labelled with conventional labelling means to act as probes or, alternatively, may be used as primers in nucleic acid amplification reactions.

Preferred nucleic acid molecules of the invention are illustrated in figures 7A, 7B, 7C, 7D, 7E and also encompass nucleic acid sequences encoding the POL protein shown in figure 7H, and the GAG protein. Sequences exhibiting at least 90 % homology with any of the afore-mentioned sequences are also comprised within the invention or fragments of any of these sequences having at least 20 and preferably at least 30 nucleotides.

The Env encoding sequence shown in figure 7C is particularly preferred, as well as the nucleic acid encoding the Env/F-S SAg protein shown in figure 7G. A further preferred nucleic acid molecule is a molecule encoding the Env/F-S Sag protein wherein the first internal stop codon (shown underlined in figure 7C), is mutated by insertion of an extra T (at position 517 in Figure 7D) to eliminate premature translational stop, the resulting sequence being then in the correct reading frame to encode the COOH terminal extension (shown underlined in Figure 7G). This protein arises naturally from read-through together with a -1 frame shift, but this process is inefficient. The synthetic 'T'-inserted cDNA provides an efficient way of producing the SAg molecule shown in Figure 7G. The single reading frame in this « synthetic » molecule thus corresponds to two different reading frames separated by a stop codon in the natural molecule.

The invention further relates to proteins expressed by human retroviruses having SAg activity and being associated with human autoimmune disease.

Fragments of these proteins having at least 6 and preferably at least 10 aminoacids are also included within the scope of the invention. Such proteins may be Gag, Pol or Env proteins or may be encoded by any Open Reading Frame situated elsewhere in the viral genome. These proteins may or may not present SAg activity. Particularly preferred proteins of the invention have SAg activity, for example that shown in Figure 7G. The protein having SAg activity may result from a premature translational stop and possibly also from a translational frameshift. A further preferred protein of the invention is the protein encoded by the synthetic cDNA and corresponding to the in-frame fusion of two normally different reading frames, together with mutation of the internal stop codon.

Endogenous retroviral ORFs typically contain a number of internal stop codons, which often render the HERV defective. It has been discovered by the present inventors that, in some cases, retroviral expression products having SAg activity result from read-through transcription of the ORF, possibly also accompanied by a reading frame shift. Consequently, the proteins exhibiting SAg activity are not, in these cases, the expected expression products of the retrovirus.

It may therefore be deduced that open reading frames of retroviruses associated with human autoimmune disease which contain at least one internal translational stop codon are among potential candidates for SAg activity. The proteins produced by premature translational stop may have an additional carboxy-terminal extension resulting from translational frame shift, for example -1 or -2 translational frame shift. Such a protein is illustrated in figure 7G.

Other proteins of the invention are those comprising the aminoacid sequences shown in figure 7D, 7F, 7H or an aminoacid sequence having at least 80 %

and preferably at least 90 % homology with the illustrated sequences or fragments of these sequences having at least 6 and preferably at least 10 aminoacids.

The invention also relates to antibodies capable of specifically recognizing a protein according to the invention. These antibodies are preferably monoclonal. Preferred antibodies are those which specifically recognize a retroviral protein having SAg activity and which have the capacity to block SAg activity. The capacity of the antibody to block SAg activity may be tested by introducing the antibody under test into an assay system comprising :

i) MHC Class II^+ cells expressing retroviral protein having SAg activity and

ii) cells bearing Vβ-T cell receptor chains of the family or families specifically stimulated by the HERV SAg expressed by the MHC Class II^+ cells, and determining the capacity of the substance under test to diminish or block Vβ-specific stimulation by the HERV SAg.

The invention also relates to cells transfected with and expressing human retrovirus having SAg activity and being associated with a human autoimmune disease. The cells are preferably human cells but may also include other type of eukaryotic cells such as monkey or other higher eukaryotes. The cells may be established cell-lines and are preferably MHC class II+.

A further important aspect of the invention relates to the identification of substances capable of blocking or inhibiting SAg activity. These substances are used in prophylactic and therapeuthic treatment of autoimmune diseases involving retroviral SAg activity.

In particular, this aspect of the invention relates to a process for identifying substances capable

of blocking or inhibiting SAg activity of a retrovirus associated with autoimmune disease, comprising introducing the substance under test into an assay system comprising :

i) MHC Class II^+ cells expressing retroviral protein having SAg activity and ;

ii) cells bearing Vβ-T cell receptor chains of the family or families specifically stimulated by the HERV SAg expressed by the MHC Class II^+ cells, and determining the capacity of the substance under test to diminish or block Vβ-specific stimulation by the HERV SAg,

The cells bearing the β-T cell receptors and the MHC Class II+ cells may be those described earlier. The substances tested in such screening procedures may be proteins, peptides, antibodies, small molecules, synthetic or naturally occurring, derivatives of the retroviruses themselves, etc...Small molecules may be tested in large amounts using combinatorial chemistry libraries.

The screening procedure may include an additional preliminary step for selecting substances capable of binding to retroviral protein having SAg activity. This additional screening step comprises contacting the substances under test, optionally labelled with detectable marker with the retroviral protein having SAg activity and detecting binding.

Once a substance or a composition of substances has been identified which is capable of blocking SAg activity, its mode of action may be identified particularly its capacity to block transcription or translation of SAg encoding sequences by :

i) contacting the substance under test with cells expressing retroviral protein having SAg activity, and

ii) detecting loss of SAg protein expression using SAg protein markers such as specific, labelled anti-SAg antibodies.

The invention also relates to a kit for screening substances capable of blocking SAg activity of a retrovirus associated with an autoimmune disease, or of blocking transcription or translation of the retroviral SAg protein, comprising :

- MHC Class II^+ cells transformed with and expressing retroviral SAg ;
- cells bearing Vβ T-cell receptor chains of the family or families specifically stimulated by the HERV SAg ;
- means to detect specific Vβ stimulation by HERV SAg ;
- optionally, labelled antibodies specifically binding to the retroviral SAg.

According to a further important aspect of the invention, there is provided a protein derived from a retroviral SAg wherein the protein is modified so as to be essentially devoid of SAg activity, thereby no longer being capable of significantly activating T-cells. Such modified proteins are however capable of generating an immune response against SAg, the immune response involving either antibodies and/or T-cells responses. The immunogenic properties of the modified proteins are thus conserved with respect with the authentic SAg.

Such modified immunogenic proteins may be obtained by a number of conventional treatments of SAg protein, for example by denaturation, by truncation or by mutation involving deletion, insertion or replacement of aminoacids.

These proteins are used in the framework of the invention as vaccines, both prophylactic and therapeutic, against autoimmune disease associated with

retroviral SAg. The vaccines of the invention comprise an immunogenically effective amount of the immunogenic protein in association with a pharmaceutically acceptable carried and optionally an adjuvant. The use of these vaccine compositions is particularly advantageous in association with the early diagnosis of the autoimmune disease using the method of the invention.

The vaccines of the invention also include nucleic acid vaccines comprising nucleic acid molecules encoding the human retroviral Sag or modified forms of the SAg known to be immunogenic but no longer active as SAgs. The nucleic acid vaccines, particularly DNA vaccines, are usually administered in association with a pharmaceutically acceptable carrier as an intramuscular injection.

The invention also relates to use of substances inhibiting either the retroviral function or the SAg function of the associated retroviruses, or Sag synthesis, in therapy for autoimmune diseases. These substances may be identified by the screening procedures described herein.

The invention further relates to methods for treatment of autoimmune diseases comprising administering an effective amount of a substance capable of inhibiting retroviral function or a substance capable of inhibiting SAg activity or synthesis.

An examples of compounds inhibiting retroviral function is AZT. Examples of compounds or substances capable of inhibiting SAg activity are antibodies to Sag, or ribozymes or antisense molecules to the SAg-encoding nucleic acid, or small molecules identifiedby virtue of their ability to inhibit SAg.

The invention also relates to a an exploratory process for detecting human autoimmune disease

associated with expression of unidentified human retrovirus Superantigen (SAg), said process comprising at least one of the following steps :

i) detecting the presence of any expressed retrovirus in a biological sample of human origin ;

ii) detecting the presence of SAg activity in a biological sample of human origin containing MHC Class II^+ cells.

This process can be used as a preliminary indication of the involvement of retroviral superantigens in autoimmune disease.

Different aspects of the invention are illustrated in the figures.

Figure 1. Leukocytes from IDDM-patients release Reverse Transcriptase (RT) activity.
(A) Supernatants derived from cultured islets isolated from two patients (Conrad et al., 1994) were assayed for RT-activity, using a half-logarithmic dilution series of purified murine leukemia virus (MLV) RT as a standard (Pyra et al., 1994). Results are expressed as mean +/- 1 SD. Islets and spleen cells from non-diabetic organ donors were cultured either alone, in the presence or absence of mitogen (-/+), or together in mixed allogeneic cultures (time as days in culture prior to collection of the supernatant is indicated below the bars).
(B) Islets and spleen cells from three non diabetic organ donors, from the two patients with acute-onset IDDM, and two patients with chronic IDDM (Conrad et al., 1994) were cultured for 1 week and supernatants were analyzed for the presence of RT-activity. Results are expressed as mean +/- 1 SD for at least three individual measurements.

Figure 2A. Isolation of a single full length retroviral genome, IDDMK$_{1,2}$22, with a six step procedure.

1) cPBS primers (Lys$_{1,2}$, Lys$_3$, Pro, Trp) were used to perform a 5' RACE 2) the eight 5' R-U5 sequences obtained in 1) were used to perform a 3' RACE with primers annealing in the R 3) the conserved RT-RNAse H region was amplified with degenerate primers 4) the 5' moiety (the predicted size for full length HERV-K-retroviruses is 3.6 kb was amplified by PCR using primers specific for the eight 5' R-U5 sequences in conjunction with a primer specific for the 3' of the central pol region obtained in step 3. The primer specific for the K$_{1,2}$22 5' consistently yielded a fragment of this size, 5) the 3' (the predicted size for HERV-K-retroviruses is 5 kb) was amplified by PCR using a primer specific for the 5' of the central pol region isolated in step 3 and primers specific for the poly(A) signals present in the 3' R-poly(A) sequences obtained in step 2. The PCR reaction using a primer specific for the 3' clone K$_{1,2}$22 (amplified in step 4) consistently yielded a fragment potentially representing an intact 3' HERV-K moiety of 5 kb, 6) the presence of an intact 8.6 kb retroviral genome containing the overlapping 5' and 3' moieties isolated in steps 4 and 5 was confirmed by PCR using primers specific for its predicted U5 and U3 regions.

Figure 2B. Consensus features of retroviral 5' end sequences (termed STRs). These consensus features are valid for retroviruses with a polyadenylation signal in the R (repeat) region. The R region is characterized by the AATAAA or ATTAAA polyadenylation signal (bold) followed by 13 to 20 nucleotides and the dinucleotide CA or GA (bold) at the 3' end of the R region. The

beginning of U5 region is defined by a GT- or T-rich sequence (underlined). The 3' end of the U5 region is in all known retroviruses defined by the dinucleotide CA, followed by one, two or three nucleotides and the primer-binding site (PB) - (N) stands for nucleotide, the suffixes x, y, and z for an undefined number.

Figure 2C. Schematic representation of mRNA-specific PCR of IDDMK$_{1.2}$-22 using a poly (A)-specific probe (Rc-T$_{(4)}$). Details of this technique are given in the « Experimental Procedure » Section of the Examples. This procedure results in a Reverse-Transcriptase-dependent amplification of retroviral genomes. The products generated can be diminished below background by RNAse treatment.

Figure 2D. Schematic representation of IDDMK$_{1.2}$-22 Provirus-specific PCR. The procedure specifically amplifies proviral 5' and 3' LTRs (long terminal repeats).
The primers used in an RT- control are substituted with either U5-primers 1) 5'ATC CAA CAA CCA Tga Tgg Ag 3' or 2) 5' TCT Cgt Aag gTg CAA Atg Aag 3' at 0.3 µM final concentration in conjunction with the U3-primers using either 3) gTA Aag gAT CAA gTg Ctg TgC 3' or 4) 5' CTT TAC AAA gCA gTA Ttg Ctg C 3' at 0.3 µM final concentration. 0.75 µl of Taq- Pwo- polymerase mix (Boehriner Mannheim, Expand™ High Fidelity PCR System) are used with a thermocycler profile corresponding to the one described for mRNA-specific RT-PCR and omitting the RT step.
Hybridization is performed with the probe and the methods corresponding those used for mRNA-specific RT-PCR.

Sequence identity is confirmed by sequencing according to standard procedures.

Figure 2E. IDDMK$_{1,2}$-22 RNA- and Provirus-specific PCR. This procedure will result in amplification products independentely of the presence or absence of RT-reactions and reflects the total retroviral RNA- and DNA- templates present in a given sample.
The same conditions as in the proviral specific PCR are used with U3 primers 1) 5'AAC ACT gCg AAA ggC CgC Agg 3' or 2) 5' Agg TAT TgT CCA Agg TTT CTC C 3' in conjunction with R (repeat) primers 3) 5' CTT TAC AAA gCA gTA TTg Ctg C 3' or 4) 5' gTA Aag gAT CAA gTg Ctg TgC 3'. Cycling conditions and primer concentrations are identical to those described for proviral specific PCR.

Figure 2F. IDDMK$_{1,2}$22 is an endogenous retrovirus found in the plasma of IDDM patients at disease onset but not in the plasma of healthy controls.
PCR primers pairs were designed that are either specific for the U3-R- or for the U3-R-poly(A)-region of IDDMK$_{1,2}$22 (see Experimental Procedures). The U3-R primer pair amplified both viral RNA and DNA, whereas the U3-R-poly(A) primer pair amplified selectively viral RNA. The amplified material was hybridized with probes generated with the molecularly cloned U3-R region of IDDMK$_{1,2}$22. Signals in the first and third rows correspond to amplification of contaminating DNA present in the plasma of IDDM patients (left hand columns, 1-10) and controls (right hand columns, 1-10) and were as expected RT-independent. In contrast, signals in the second row resulted from the amplification of viral RNA present only in IDDM

patients (left hand columns, 1-10) but not in the non diabetic controls (right hand columns, 1-10). This was supported by the absence of amplification products in reactions lacking RT (fourth row, right and left hand clumns, 1-10). In addition the signal could be diminished below background by RNAse treatment (data not shown). In the fifth row the genomic DNA from IDDM patients and controls was amplified with the U3-R-specific primers. The primer pair specific for the U3-R-poly(A), in turn, did not result in amplification of genomic DNA (data not shown).

Figure 3. Phylogenetic trees of coding and non-coding regions place IDDMK$_{1,2}$22 in the HERV-K10 family of HERVs.

(A) IDDMK$_{1,2}$22 SU-ENV is most closely related to HERV-K10, and is also related to the B-type retroviruses MMTV and JSRV.

(B) The phylogenetic analysis of the RT region shows that IDDMK$_{1,2}$22 belongs to the HERV-K10 family and is more closely related to B-type retroviruses such as MMTV than to D-type retroviruses such as Simian Mason Pfizer (SMP) or Spumaviridae (SFV). Abbreviations used: SRV-2, Simian retrovirus; JSRV, Jaagsiekte Sheep retrovirus; SFV; Simian foamy virus).

(C) The non-coding LTR region was used to construct a phylogenetic tree of the HERV-K family. K$_{1,2}$1 and K$_{1,2}$4 (see above) were isolated only as subgenomic or truncated transcripts. K$_{1,2}$1 is related to KC4, while K$_{1,2}$4 and IDDMK$_{1,2}$22 are related to the K10/K18 subfamily. Within this family, K$_{1,2}$4 is closely related to K10, whereas IDDMK$_{1,2}$22 appears to be more distant.

Figure 4. The pol-env-U3-R region of IDDMK$_{1,2}$22 exerts an MHC class II dependent but not MHC restricted mitogenic effect upon transfection in monocytes.

(A). IDDMK$_{1,2}$22 is expected to generate two singly spliced subgenomic RNAs, one encoding ENV, and one comprising the U3-R region. The episomal expression vector was engineered to carry a proximal SD downstream of the promoter (pPOL-ENV-U3). Thus, the two naturally expected subgenomic RNAs can also be generated.

(B) Monocytic cell lines do not express MHC class II surface proteins in the absence of induction by Interferon-g (INF-g), (reviewed by Mach et al., 1996). The monocyte cell line THP1 was transiently transfected with pPOL-ENV-U3 or with the expression vector alone (pVECTOR). Mitomycin C treated transfectants, either induced with INF-g for 48 h or non-induced (+/- INF-g, indicated below the x-axis) were cultured with MHC-compatible T cells at different responder : stimulator ratios as indicated below the graphs (T : APC). ^{3}H-Thymidine incorporation was measured during the last 18 h of a 72 h culture and is given on the y-axis as n x 10^{3} cpm. Results are presented as mean +/- 1 SD.

(C) The MHC class II transactivator CIITA mediates INF-g inducible MHC class II expression (reviewed by Mach et al., 1996). An integrative and stable THP1-CIITA transfectant (THP1-CIITA) was transfected with pVECTOR or pPOL-ENV-UR and was used in functional assays identical to those described in Figure 4B.

(D) Peripheral blood lymphocytes (PBL) from healthy, MHC-unrelated donors (donors I, II and III indicated below the x-axis) were cultured with retroviral (pPOL-ENV-U3) and control transfectants (pVECTOR) at T : non - T ratios as indicated below the graphs (T : APC).

Figure 5. IDDMK$_{1,2}$22 mediates a Vb 7-specific SAG-effect.
10^6 T cells/ml were cultured for 3 days with Mitomycin-treated pPOL-ENV-U3 and pVECTOR transfectants at T : non - T ratios as indicated. Twenty U/ml of recombinant IL-2 were then added to the cultures and FACS analysis performed after 3 to 4 days of expansion (Conrad et al., 1994).
(A) THP1 cells were transfected with pPOL-ENV-U3, the stimulated and expanded T cells were stained with anti-CD3 monoclonal antibodies and an isotype control after 7 days of coculture.
(B) T cells stimulated by THP1 transfected with the vector (pVECTOR) alone were stained with anti-CD3 monoclonal antibodies and the anti Vb 7-specific antibody 3G5.
(C) THP1 cells were transfected with pPOL-ENV-U3, the stimulated T cells were stained with anti-CD3 monoclonal antibodies and the anti Vb 7-antibody 3G5.

Table1. IDDMK$_{1,2}$22 mediates a Vb 7-specific SAG-effect. The B lymphoblastoid cell line Raji was stably transfected with either pPOL-ENV-U3 or pVECTOR, and used in functional assays (equivalent to Figure 5) 2 weeks after selection. The monocytic cell line THP1 was cultured for 48 hours after transfection with the same constructs. The percentages of double positive (CD3 and Vb-7, Vb-8, -12) T cells are indicated that were obtained after 1 week of coculture with the respective transfectants (pPOL-ENV-U3 or pVECTOR).

Figure 6. The N-terminal env moiety of IDDMK$_{1,2}$22 mediates the SAG-effect.
(A). Based on the construct pPOL-ENV-U3 different deletional mutants were generated that comprised 1)

pPOL: the pol gene; 2) pPOL-ENV/TR: the pol -.and the N-terminal moiety of the env-gene; 3) pCI-ENV/TR: the N-terminal moiety of env-gene alone.
(B). PBL from MHC unrelated donors were cocultured with Mitomycin C treated THP1 cells as described in Figure 4. The individual transfectants are indicated with the names of the constructs above the bars. (1) pVECTOR, 2) pPOL, 3) pPOL-ENV-U3, 4) pPOL-ENV/TR, 5) pCI-neo, 6) pCI-ENV/TR). One of at least three independent ^{3}H-Thymidine incorporation experiments with allogeneic T cells stimulated by the individual transfectants is shown. The ratio between T cells and transfectants is indicated below the bars (T : APC).

Figure 7A. IDDMK$_{1,2}$22 - 5' LTR.
This figure shows the sequence of the 5' LTR (U3 RU5) of the IDDMK$_{1,2}$22 - provirus.

Figure 7B. IDDMK$_{1,2}$22 - 3' LTR.
This figure shows the sequence of the 3' LTR (U3 RU5) of the IDDMK$_{1,2}$22 provirus.

Figure 7C. IDDMK$_{1,2}$22 - env.
This figure shows the full nucleotide sequence of the env coding region, starting with the ATG initiate codon at position 59 (as shown in Figure 7D).
The first internal stop codon TAG at position 518 is underlined corresponding to the codon where following a -1 frame shift, translation stops to give rise to the protein illustrated in Figure 7D.
The second internal stop codon TAG at position 601 (in frame with the earlier TAG) is also underlined. Translational stop at this codon gives rise to the

IDDMK$_{1.2}$22 - ENV / FS (SAG) protein illustrated in Figure 7G. The nucleic acid coding for the IDDMK$_{1.2}$22 - env/fs (SAG) protein is also shown in Figure 7E.

Figure 7D. The nucleotide and deduced amino acid sequence of IDDMK$_{1.2}$22-SAG.
The minimal stimulatory sequence corresponding to the insert of pCI-ENV/TR comprises a C-terminally truncated protein of 153 amino acids. There is only one ORF with a stop codon at position 518. The first potential start codon in a favorable context is at position 59. Two potential N-linked glycosilation sites are present at positions 106, and 182 respectively. The degree of homology with other retroviral ENV proteins is shown in Figure 3A. No significant homology was detected with the SAG of MMTV or with autoantigens known to be important in IDDM.

Figure 7E. IDDMK$_{1.2}$22 - env/fs - sag.
Nucleotide sequence coding for the 181 amino acid IDDMK$_{1.2}$22 - ENV/FS - SAG protein shown in Figure 7G and corresponding to a read-through translation of the first stop codon at 518 together with a -1 frame shift.

Figure 7F. IDDMK$_{1.2}$22 - ENV.
Deduced amino acid sequence encoded by the full env coding region (as shown in Figure 7B), without frame shift.
The underlined « Z » is the stop site for the 153 amino acid protein shown in Figure 7D.

Figure 7G. IDDMK$_{1.2}$22 ENV/FS (SAG).

With respect to wild-type IDDMK$_{1.2}$22 env an insertion of a T at position 517 (underlined) results in a predicted protein corresponding to the one expected to be generated by IDDMK$_{1.2}$22 ENV/FS. The additional predicted C terminal amino acids that characterize ENV-FS are underlined. This protein has marked SAg activity.

Figure 7H. IDDMK$_{1.2}$22 POL.
Deduced amino acid sequence of the POL protein of IDDMK$_{1.2}$22.

Figures 8A to 8G illustrate candidate 5' STRs isolated in the first step of the six-step procedure (illustrated in Figure 2A) to isolate putative retroviral genomes from IDDM patients.

Figure 9. Functional assay for the presence of Vβ7-IDDM-SAG in PBL.
PBL (peripheral blood lymphocytes) are isolated from 10ml of Heparine-blood (Vacutainer) from IDDM patients or controls with Ficoll-Hypaque (Pharmacia).
5 x 10^6 PBL are incubated with or without 10^3 U/ml recombinant human INF-γ (Gibco-BRL) for 48 hours.
100 µg/ml Mitomycin C (Calbiochem) are added to inactivate for 10^7 cells for 1 hour at 37°C, and extensive washing is performed.
Culture with T cell hybridomas bearing human Vβ-2, -3, -7, -8, -9, -13 and -17 at stimulator : responder ratios of 1 : 1 and 1 : 3 in 96 round bottom wells.
TCR-crosslinking with anti-CD3 antibodies (OKT3) is used as a positive control for each individual T hybridoma.

IL-2 release into the supernatant is measured with the indicator cell line CTLL2 according to standard procedures.
Results are expressed as percentage of maximal stimulation obtained with TCR crosslinking in the same experiments.
A selectively induced TCR-crosslinking and IL-release of Vβ7 is interpreted as being compatible with the presence of IDDM-SAG in PBL from the individual analysed.

EXAMPLES

In two patients with type I diabetes, a dominant pancreatic enrichment of one Vb-family, Vb 7, has been observed (Conrad et al., 1994). The same dominant enrichment of Vb 7 could be mimicked by stimulating T cells of diverse haplotypes with surface membrane preparations derived from the pancreatic inflammatory lesions but not with membranes from MHC-matched healthy control islets. This was taken as evidence for the presence of a surface membrane-associated SAG (Conrad et al., 1994).

In the framework of the present invention, the hypothesis that this SAG is of endogenous retroviral origin has been tested. Below it is shown that the SAG identified in these two patients is encoded by a human endogenous retrovirus related to MMTV. Expression of this endogenous SAG in IDDM suggests a general model according to which self SAG-driven and systemic activation of autoreactive T cells leads to organ-specific autoimmune disease.

Example 1. Cultured leukocytes from inflammatory b-cell lesions of IDDM-patients release Reverse Transcriptase activity

Expression of cellular retroelements may be associated with measurable Reverse Transcriptase-activity (RT) (Heidmann et al., 1991). An RT-assay detected up to a hundredfold increase in RT-activity in supernatants from short-term cultures of freshly isolated pancreatic islets derived from two patients (Figure 1A), (Conrad et al., 1994; Pyra et al., 1994). No RT-activity above background levels was detected in medium controls, indicating that the RT-activity could not be accounted for by a contamination of the synthetic media and sera with animal retroviruses. We can also exclude the possibility that the RT-activity represents cellular polymerases released into the supernatant by dying cells. Indeed, no RT-activity can be detected in cultures from non-diabetic controls under conditions in which cell death is strongly enhanced, namely mitogen treated peripheral blood lymphocytes (PBL), splenocytes and cocultures of islets with allogeneic T cells. Moreover, the IDDM-derived islets were cultured for 5 days, whereas control cultures were sequentially analysed for up to 4 weeks. Finally the absence of RT-activity in the supernatants of the mitogen-treated control PBL also excluded the possibility that the RT-activity detected with the IDDM islets was simply due to non-specific cell activation. Both, the islets and the inflammatory infiltration represented potential sources for the enzymatic activity. As shown in Figure 1B, supernatants from cultured spleen cells from the patients contained more RT-activity than the inflammatory b-cell lesions. Moreover, the RT-activity disappeared together with the local inflammatory lesion in two patients with chronic and long-standing disease,

but it persisted in cultured spleen cells from the same patient (Figure 1B). This was interpreted as being compatible with the leukocytes as the most likely source of this RT-activity.

Example 2. Isolation of a full length retroviral genome, IDDMK$_{1,2}$22, from supernatants of IDDM islets

A strategy to isolate putative retroviral genomes from polyadenylated RNA extracted from the supernatants of IDDM islets was developed (Figure 2A). This strategy relies on the following three characteristic features of functional retroviruses. First, retroviral genomes contain a primer binding site (PBS) near their 5' end. Cellular tRNAs anneal to the PBS and serve as primers for Reverse Transcriptase (reviewed by Whitcomb and Hughes, 1992). Second, the R (repeat) sequence is repeated at the 5' and 3' ends of the viral RNA (Temin, 1981). Third, the RT-RNAse H region of the pol gene is the most conserved sequence among different retroelements (McClure et al., 1988; Xiong and Eickbusch, 1990). These three features were exploited in a six step procedure as follows.

1) To isolate the 5' ends (5'R-U5) of putative retroviral RNA genomes, a 5' RACE procedure was performed with primers complementary to known PBS sequences (cPBS primers) (Weissmahr et al., 1997). Most retroviruses known have a primer binding site (PBS) complementary to one of only four individual 3' ends of tRNAs : $tRNA^{Pro}$, $tRNA^{Lys3}$, $tRNA^{Lys1.2}$ and $tRNA^{Trp}$. Accordingly, sequence-specific primers complementary to the four PBSs were used to derive cDNA (Weissmahr, 1995). The amplification products resulting from anchored PCR and of 100 - 700 bp in size were sequenced and analyzed for the presence of consensus sequences

typically found in retroviral 5' R-U5s (Weissmahr, 1995).

Eight different candidate 5'R-U5 sequences (5'$K_{1,2}$-1, -4, -10, -16, -17, -22, -26 and -27) were obtained with the cPBS-$Lysine_{1,2}$ primer. All eight sequences contained features typical of the 5' ends of retroviral genomes (Temin, 1981). These include the presence at the expected positions of i) a PBS region, ii) conserved and correctly spaced upstream regulatory sequences, such as a poly(A) addition signal and site, and the downstream GT- or T - rich elements (Wahle and Keller, 1996), iii) a putative 5' end specific U5 region and iv) a putative R region. Of the eight 5' R-U5 sequences isolated, three (5'$K_{1,2}$-1, -4,and -22) were identified on the basis of sequence homology as belonging to previously identified families of human endogenous retroviruses (HERVs) that are closely related to mouse mammary tumour viruses (MMTV), namely HERV-K(C4) (Tassabehji et al., 1994), HERV-K10 and HERV-K18 (Ono, 1986a; Ono et al., 1986b). The remaining five sequences exhibited only a distant relationship with HERV-K retroviruses.

2) A repeat (R) region conserved in the 5' R-U5 and the 3' U3-R-poly(A) is essential for retroviral first strand DNA synthesis to proceed to completion (Whitcomb and Hughes, 1992). Primers specific for the R region-sequence obtained for individual 5' R-U5s were used to prime the cDNA synthesized with oligo(dT), (Weissmahr, 1995). Products resulting from anchored PCR were sequenced and analyzed for the presence of a conserved R region followed by a poly(A)-tail. The eight 3'R-poly(A) ends (3'$K_{1,2}$-1, -4, -10, -16, -17, -22, -26 and -27) corresponding to the eight different 5'R-U5 regions identified in step 1 were isolated by means of a 3' RACE procedure using primers specific for the R regions. In each case, the isolated sequences

contained the expected R region followed by a poly(A) tail.

3) The conserved RT-RNase H region within the pol gene was next amplified by PCR using degenerate primers (Medstrand and Blomberg, 1993). 15 individual subclones were sequenced and all exhibited approximately 95% similarity at the protein level to the RT-RNase H region of the HERV-K family.

4) The 5' moiety (from the U5 region at the 5' end to the pol gene) of the putative retroviral genome was amplified by PCR using primers specific for the eight different U5 regions present in the 5'R-U5 sequences (isolate in step 1) in conjunction with a primer specific for the 3' end of the central pol region (isolated in step 3). The expected size of the PCR product corresponding to the 5' moiety of full length HERV-K retroviruses is 3.6 kb (Ono et al., 1986b). Only the PCR reaction using the primer specific for the $K_{1,2}22$ 5' end clone consistently yielded a fragment of this size. Sequence analysis of several independent clones confirmed that this 3.6 kb fragment contains the R-U5-PBS region followed by coding regions corresponding to the gag and pol genes, and thus indeed represents the 5' moiety of an intact retroviral genome.

5) The 3' moiety (from the pol gene to the 3' end) of the putative retroviral genome was amplified by PCR using a primer specific for the 5' end of the central pol region (isolated in step 3) and primers specific for the poly(A) signals present in the 3'R-poly(A) sequences (isolated in step 2). The expected size of the PCR product corresponding to the 3' moiety of full length HERV-K-retroviruses is 5 kb (Ono et al., 1986b). The PCR reaction using a primer specific for the 3' end clone $K_{1,2}22$, which is the one that should correspond to the 3' end of the retrovirus from which the 3.6 kb

5' moiety was amplified in step 4, consistently yielded a fragment potentially representing an intact 3` moiety of 5 kb. Sequence analysis of several independent clones confirmed that this 5 kb fragment indeed contains coding regions corresponding to the pol and env genes followed by the expected U3-R-poly(A) region.

6) Finally, the presence of an intact 8.6 kb retroviral genome containing the overlapping 5' and 3' moieties isolated in steps 4 and 5 was confirmed by PCR using primers specific for its predicted U5 and U3 regions.

The full length retroviral genome that was isolated was called IDDMK$_{1,2}$22, where IDDM refers to the tissue source, K$_{1,2}$ refers to Lysine$_{1,2}$ cPBS primer and 22 represents the serial number of the clone. IDDMK$_{1,2}$22 was determined to be novel retrovirus on the basis of two criteria. First, it has a unique pattern of restriction enzyme cleavage sites that is distinct from that of other known viruses. Second, its nucleotide and amino acid sequences in non-coding and coding regions diverge from other known retroviruses by at least 5-10 %.

IDDMK$_{1,2}$22 was the only full length virus identified in these experiments, suggesting that it is the only functional retrovirus specifically associated with the supernatants of the cultured IDDM islets. PCR reactions using primers specific for the other 5'R-U5-PBS and 3'U3-R-poly(A) clones isolated in steps 1 and 2 did not yield fragments of the size expected for intact retroviral genomes in steps 4 and 5. In particular, primers specific for the 5' and 3' ends corresponding to the ubiquitous HERV-K10 virus did not amplify fragments corresponding to complete genomes, although this virus is known to be released as full length genome associated with viral particles from several cell lines and tissues (Tönjes et al., 1996). Our

inability to detect full length HERV-K10 genomes in the IDDM islet supernatant is unlikely to be due to a technical problem because it could be amplified very efficiently from both genomic DNA and a size selected cDNA library prepared from a B-lymphoblastoid cell line (data not shown). It is more likely that HERV-K10 is not released in significant amounts by the cultured IDDM islets.

Finally, i) we confirmed by RNA-specific PCR that sequences identical, or highly similar, to the 3' U3-R-poly(A) of $IDDMK_{1,2}$ were present in RT-positive but not in RT-negative samples analysed; ii) in a preliminary epidemiological study we detected by PCR sequences identical, or highly similar, to the 3' U3-R-poly(A) of $IDDMK_{1,2}$ only in the plasma of 10 recent onset IDDM patients but not in the plasma of 10 age-matched non diabetic controls (Figure 2F); and iii) we confirmed by PCR the presence of sequences identical, or highly similar to the U3-R region of $IDDMK_{1,2}$ in genomic DNA of IDDM patients (n = 10) and non diabetic controls (n = 10) (Figure 2F). In summary, these data indicate that $IDDMK_{1,2}$ is an endogenous retrovirus that is released from leukocytes in IDDM patients but not in non diabetic controls.

Example 3. $IDDMK_{1,2}22$ is a novel member of the MMTV-related family of HERV-K, and is related to HERV-K10

To evaluate the relationship between $IDDMK_{1,2}22$ and other known retroviruses we derived phylogenetic trees for subregions exhibiting different degrees of conservation (Galtier et al., 1996; Saitou and Nei, 1987; Thompson et al., 1994). The three regions chosen for this analysis were the RT region of the pol gene

(Figure 3B), the outer region (SU, surface) of the env gene (Figure 3A) and the U3 region of the LTR (Figure 3C). The RT and SU regions were selected to construct interspecies phylogenetic trees because they represent, respectively, the most highly conserved and the most variable of the protein coding regions (McClure et al., 1988). The U3 region of the LTR was chosen to construct an intraspecies tree of the family to which IDDMK$_{1,2}$22 belongs because LTR sequences are conserved in size and sequence only within a given species, and the U3 region accounts for most of the intraspecies differences (Temin, 1981). As shown in Figure 3A, the ENV polyprotein of IDDMK$_{1,2}$22 is most closely related to that of HERV-K10. Both proteins are related to those of MMTV and Jaagsiekte sheep retrovirus (JSRV). The same is essentially true for the RT-subregion of the POL polyprotein, where IDDMK$_{1,2}$22 and HERVK10 are most closely related to the B-type retrovirus MMTV (Figure 3B). Figure 3C illustrates, that K$_{1,2}$1 is related to HERV-K(C4), while K$_{1,2}$4 and IDDMK$_{1,2}$22 are related to the K10/K18 subfamily. Within this family, K$_{1,2}$4 is closely related to K10, whereas IDDMK$_{1,2}$22 appears to be more distant.

Example 4. IDDMK$_{1,2}$22 encodes a Vb7-specific SAG

The strategy used to identify a putative SAG-function encoded by IDDMK$_{1,2}$22 was dictated by 1) predictions based on the biology of the MMTV-SAG, 2) general requirements for a protein-protein interaction between a SAG and MHC class II molecules and 3) intracellular trafficking mechanisms used by proteins encoded by retroviruses. The prototypical retroviral SAG of MMTV is a type II transmembrane protein that is encoded within the U3 of the 3' LTR (reviewed by Acha-

Orbea and McDonald, 1995). It is targeted into the MHC class II peptide loading compartment and exported to the cell surface. On the basis of potential splice donor (SD) and acceptor sites (SA) present in its sequence, IDDMK$_{1,2}$22 is expected to generate two subgenomic mRNAs, one encoding ENV and a second transcript comprising the U3-R region (Figure 4A). Based on these criteria we produced an episomal expression construct (pPOL-ENV-U3) with a 5' SD positioned upstream of the truncated pol, env and U3-regions (Figure 4A). It is expected that both of the putative subgenomic mRNAs can be generated from this construct (Figure 4A).

Retroviral- and control-transfectants of monocyte- and B lymphocyte-cell lines were generated and tested for their ability to stimulate MHC compatible and allogeneic T cell lines in a Vb7-specific manner. Monocytes do not express measurable MHC class II surface proteins in the absence of induction by Interferon-g (INF-g); the MHC class II transactivator CIITA mediates INF-g-inducible MHC class II expression (reviewed by Mach et al., 1996). As shown in Figure 4A, transient monocyte (THP1, U937) transfectants induced with INF-g and expressing the truncated IDDMK$_{1,2}$22 genome (pPOL-ENV-U3) stimulated in a dose-dependent fashion T cell lines from

MHC-compatible donors essentially to the same extent. The mitogenic effect was dependent on the presence of MHC class II, since INF-g-mediated MHC class II expression specifically induced the stimulatory capacity of retroviral- as compared to control-transfectants (Figure 4B). The use of THP1 cells rendered constitutively MHC class II positive by transfection with CIITA resulted in a stimulation comparable to INF-g-induction, suggesting that the INF-g-induced and CIITA-dependent MHC class II expression

was indeed responsible for this functional difference (Figure 4C). The mitogenic effect is not MHC-restricted, since a response exceeding allostimulation was observed when PBL from several different MHC-disparate donors were tested for proliferative responses to monocytes transfected with pPOL-ENV-U3 (Figure 4D). In essence, these functional data suggest that the truncated IDDMK$_{1,2}$22 (pPOL-ENV-U3) genome is responsible for a mitogenic effect that is MHC class II-dependent but not MHC-restricted.

Experiments were performed in bulk-cultures using TCR-Vb-specific stimulation and expansion as a readout. Retroviral THP1 transfectants induce a more than 15 fold increase in the number of the Vb-7 family but not of the two control families tested (Vb8, Vb12) after specific stimulation and subsequent amplification (Figure 5, Table 1). This was verified by using two different Vb-7-specific monoclonal antibodies, 3G5 and 20E. A comparable effect was also observed when PBL from MHC-disparate donors were tested. This was interpreted as evidence for the presence a Vb-7-specific SAG.

The monocytic cell lines were at least 3 times more efficient in terms of specific TCR Vb-7 amplification as compared to the most efficient B lymphoblastoid cell line (Table 1). This difference could not be explained by variations in the level of MHC class II expression or by the individual MHC haplotypes present. On the other hand, it may be due to differential expression of costimulatory molecules or secretion of cytokines. In conclusion, by all criteria known to date, IDDMK$_{1,2}$22 encodes a mitogenic activity having all features of a Vb-7-specific SAG.

TABLE 1 : IDDMK$_{1,2}$22 mediates a Vβ7-specific SAG-effect

TRANSFECTANT	Vβ-FAMILY		
	Vβ-7	Vβ-8	Vβ-12
Raji-pPOL-ENV-U3	7%	5%	2.5%
Raji-pVECTOR	1.5%	5.5%	2%
THP1-pPOL-ENV-U3	16%	5.3%	2.8%
THP1-pVECTOR	1%	5.8%	3%

Example 5. The SAG function is mediated by the N-terminal moiety of the env protein

A series of deletional mutants were generated that contained either the truncated pol-env-U3 region (pPOL-ENV-U3), the truncated pol gene alone (pPOL), or the truncated pol gene followed by the env gene truncated downstream of the premature stop codon found in all clones (pPOL-ENV/TR), (Figure 6A). In addition, a C-terminally truncated env gene was generated as an individual expression unit (pCI-ENV/TR). As shown in Figure 6B, by excluding the env-coding region the SAG-function is selectively lost (pPOL). If, however, the truncated env gene is included (pPOL-ENV/TR), the stimulatory capacity is restored to levels comparable to pPOL-ENV-U3. In addition, expression of the truncated env gene alone (pCI-ENV/TR) is sufficient for function. These findings demonstrate that the SAG

function is mediated by the N-terminal moiety of the env gene comprising 153 amino acids. The nucleotide and predicted amino acid sequences of the minimal stimulatory region are shown in Figure 7. As shown in Figure 3A, this predicted protein resembles the N-terminal ENV proteins of related HERVs (HERV-K10), and those of the B-type retroviruses (MMTV, JSRV). However, there is no significant sequence homology with either MMTV-SAG, other SAGs, or autoantigens known to be important in IDDM.

Here, evidence is provided showing that a human endogenous retrovirus, $IDDMK_{1,2}22$, is released from leukocytes in patients with acute onset type I diabetes. In preliminary experiments $IDDMK_{1,2}22$ RNA sequences were detectable in the plasma of IDDM patients at disease onset but not in the plasma of age-matched healthy controls. This novel human retrovirus is related to MMTV and encodes a SAG with functional characteristics similar to the one encoded by MMTV. In contrast to MMTV, however the IDDM-associated SAG is encoded within the retroviral env gene rather than within the 3' LTR. It has the same TCR Vb7-specificity with the SAG originally identified in the IDDM patients. This SAG is thus likely to be the cause of the Vb7-enriched repertoire of islet-infiltrating T lymphocytes.

$IDDMK_{1,2}22$ as a member of the HERV-K class of endogenous retroviruses

HERV-K genomes exist in two different forms, type I genomes which are largely splice deficient and type II genomes which generate three subgenomic mRNAs (Tönjes et al., 1996; Ono, 1986). A 292 bp insert at the pol-

env boundary with clustered nucleotide changes downstream of the splice acceptor site are present in type II but not in type I genomes (Tönjes et al., 1996). The insert affects both, the env and pol gene: i) type II genomes have a stop codon between env and pol which is missing in type I genomes and ii) have a considerably longer N terminal env region. The 292 bp insert and the clustered nucleotide changes have been proposed to be responsible for the efficient splicing of type II genomes (Tönjes et al., 1996). IDDMK$_{1,2}$22 is missing the 292 bp insert but has two in frame stop codons between env and pol and the clustered nucleotide changes downstream of the SA typical of those found in type II genomes. In terms of splice efficiency, IDDMK$_{1,2}$22 may be in an intermediate position between type I and II genomes. This and the altered N terminal sequences in IDDMK$_{1,2}$22 with respect to type II genomes may affect SAG expression in vivo. However, as shown in Figure 4, the 3' terminal moiety (POL-ENV-U3) of the IDDMK$_{1,2}$22 genome mediates the SAG function in vitro. Moreover, it is known from MMTV that the SAG function in vivo may be present at levels where the respective protein remains undetectable (Winslow et al., 1992; reviewed by Acha-Orbea and MacDonald, 1995).

The model: human self SAGs as activators of autoreactive T cells in type I diabetes

A model is proposed according to which induction of self SAGs in systemic and professional APCs, outside the pancreas, leads to autoimmunity in genetically susceptible individuals. The model implies two steps, the first is systemic, the second organ-specific. The initial event is a systemic, polyclonal activation of a Vb-restricted T cell subset, triggered by the

expression of an endogenous retroviral SAG in professional MHC class II$^+$APCs. In a second step, autoreactive T cells within the subset of SAG-activated T lymphocytes initiate organ-specific tissue destruction. The evidence presented here, however, does not rule out that the release of the IDDMK$_{1,2}$22 RNA sequences in vivo and the SAG function associated with IDDM in these patients are the consequence rather than the cause of the inflammation.

The expression of self SAGs can in principle be modulated by two variables: physiological endogenous stimuli or environmental stimuli. A possible physiological stimulus might be steroid hormones. HERV-K10 expression is steroid-inducible in vitro and this is possibly the result of hormone response elements (HRE) present in its LTR (Ono et al., 1987). IDDMK$_{1,2}$22 and HERV-K10 share the same putative HRE in their respective LTRs (Ono et al., 1987), (Figure 3). Steroid inducibility of IDDMK$_{1,2}$22 could therefore also occur in vivo, in analogy to the well documented example of the transcriptional control by steroid hormones of the MMTV promoter (reviewed by Acha-Orbea and Mac Donald, 1995). Infectious agents are of major importance when considering environmental factors. Examples include the cellular SAGs that are expressed by herpesvirus-infected monocytes and B-lymphocytes (Dobrescu et al., 1995; Sutkowski et al., 1996). In both cases, HERVs have not been excluded as a potential source of the SAG-activity. It is thus conceivable that SAGs are being selectively expressed in response to ubiquitous pathogens such as herpesviridae (reviewed by Roizman, 1996). In fact, HERVs are induced by a variety of environmental stresses, and some of them behave as hepatic acute-phase genes (reviewed by Wilkinson et al., 1994).

The experimental evidence presented suggests that the RT-activity, the IDDMK$_{1,2}$22 RNA sequences and in consequence the SAG may derive from leukocytes rather than from the pancreatic b-cells. This may indicate that expression of the retroviral SAG is induced preferentially in systemically circulating professional MHC class II$^+$ APCs. The highest rate of IDDM coincides with puberty (10-14 years) in both sexes (Bruno et al., 1993). Infections with ubiquitous viruses (reviewed by Roizman, 1996) may act synergistically with an increase in the circulating levels of steroids to enhance expression of the SAG in professional APCs. Autoreactive T cells can be readily demonstrated in the mature repertoire of healthy individuals (Pette et al., 1990). However, in order to able to migrate to the target tissue these T cells have to be activated (reviewed by Steinman, 1995). These considerations lead us to the hypothesis that among the Vb7$^+$-T cells activated by IDDMK$_{1,2}$22-SAG, some are autoreactive and migrate to the target tissue were b-cell specific death ensues. Once b-cells die, cellular antigens are liberated and the immune response perpetuated through determinant spreading (reviewed by McDevitt, 1996).

The concept of IDDMK$_{1,2}$22-sag as autoimmune gene

Known genes conferring susceptibility to autoimmune diseases are host-derived, stably inherited Mendelian traits and contribute in a cumulative fashion to the familial clustering of the disease without causing disease per se (reviewed by Todd, 1996). IDDMK$_{1,2}$22 should be viewed as mobile genetic element with the potential to move within the host genome due to multiple mechanisms, including retrotransposition,

homologous recombination, gene conversion and capture, resulting in multiple copies of individual HERVs (reviewed by Preston and Dougherty, 1996; Wain-Hobson, 1996). This renders family studies dealing with searches for HERV-disease association difficult. It should be noted, however, that there is little or no plus / minus genetic polymorphism in different humans at the HERV-K loci and as yet no evidence for mobility. Interestingly, an IDDMK$_{1,2}$22-related HLA-DQ-LTR is associated with susceptibility to IDDM, possibly due to cosegregation with the HLA (Figure 3C), (Badenhoop et al., 1996). In addition, infectious transmission cannot be excluded, as is the case for two closely related virus groups containing endogenous and exogenous variants: MMTV and JSRV (Figure 4A and 4B), (reviewed by Acha-Orbea and McDonald, 1995; York et al., 1992).

In summary, this candidate autoimmune-gene has distinctly different features from classical, disease-associated susceptibility genes. It has the potential of being transmitted as either an inherited trait or as an infectious agent. Moreover, this gene has no apparent essential function for the host but it may have instead an inducible and intriguing potential to directly cause disease whenever expressed in genetically susceptible individuals.

Experimental Procedures

Patients

The the islets and spleens from patients with acute onset- and chronic IDDM and non diabetic organ donors were provided by the Pittsburgh Transplant Institute (Conrad et al., 1994).

The plasma and genomic DNA from patients and controls for the epidemiological study were isolated by the Diabetes Register in Turin, Italy (Bruno et al., 1993). The samples were collected within 1 month after the clinical diagnosis from patients, aged from 0 - 29 years (Bruno et al., 1993).

RT assays

RT assays were performed as described (Pyra et al., 1994).

Isolation of full length retroviral genomes

A description of the criteria used to identify unknown retroviral 5' R-U5s and 3' R-poly(As) has been published (Weissmahr et al., 1997).

I. Primers sequences for the 3'moiety of the putative retroviral genomes; abbreviations are according to Eur. J. Biochem. (1985). 150, 1-5.

A. RT region

RT 1a 5'YAAATggMgWAYgYTAACAgACT3'

RT 1b 5'YAAATggMgWAYgYTAACTgACT3'

RT 2a-nested

5'CgTCTAgAgCCYTCTCCggCYATgATCCCg3'

RT 2b-nested

5'CgTCTAgAgCCYTCTCCggCYATgATCCCA3'

B. 3' U3-R-Poly(As): all primers have an identical 5'-anchor:

5'TgCgCCAgCAATgTATCCATg3'+ sequence-specific part

#1K1,2-1 5'gggTggCAgTgCATCATAggT3'

#4K1,2-4 5'gggAgAgggTCAgCAgCAgACA3'

#K1,2-10 5'gACAgCAAgCCAgTgATAAgCA3'

#K1,2-16 5'ggAACAgggACTCTCTgCA3'

#K1,2-17 5'gggAAgggTAAggAAgTgTg3'

#K1,2-22 5'ggTgTTTCTCCTgAgggAg3'
#K1,2-26 5'gAAgAATggCCAACAgAAgCT3'
#K1,2-27 5'gggAAACAAggAgTgTgAgT3'
common, secondary anchor primer:
3' U3-R-poly(As)common

5'CATgTATATgCggCCgCTgCgCCAgCAATgTATCC
ATgg3'

II. Primer sequences for the 5' moiety of the genome:
A. RT-region
RT 1 5'TATCTTTCgTTTCTgCAgCAC3'
RT 2 5'TAACTggTTgAAgAgCTCgACC3'
B.5'-R-U5
R-U5-1 5'ATACTAAggggACTCAgAggC3'
R-U5-2 5'CAgAggCTggTgggATCCTCCATATgC3'

The PCR conditions were as follows: 1x 94° C 2 min; 45° C 5 min; 68° C 30 min; 10x 94° C 15 sec; 45° C 30 sec + 1° C/cycle; 68° C 3 min 30 sec; 25x: 94° C 15 sec; 55° C 30 sec; 68° C 3 min 30 sec + 20 sec/cycle. Primers were used at 300 nM final concentration, dNTPs at 200mM, with 52 U/ml of Taq-Pwo polymerase-mix (Boehringer Mannheim). One vol% of first-round PCR was subjected to a nested PCR. Size selected and purified amplification products were blunted, EcoRI adapted and subcloned into EcoRI-digested lZAPII-arms. After two rounds of hybridisation 20 individual clones were rescued as plasmids. Eleven clones were selected for further analysis based on a conserved restriction pattern.An equivalent procedure was followed for the 5' moiety of the genome. Sequencing was performed on an automatic sequencer (ABI, Perkin Elmer) using subgenomic clones.

Epidemiological study. RNA-PCR. Three ml of blood was collected in EDTA tubes (Vacutainer) and further processed within 6 hours. Samples were subjected twice to centrifugation, for 4 x 10^3 G, 10 min at 4°C. Total RNA was extracted from 560 ml of plasma (QIAamp; Qiagen). Four vol % of total RNA was used for a single tube RT-PCR using thermostable AMV, Taq and Pwo (Boehringer Mannheim). Reactions contained at a final concentration: di-Na salts of dNTPs at 0.2 mM; DTT at 5 mM; 10 U recombinant RNAsin (Promega); 1.5 mM $MgCl_2$; R-poly(A) primer 5' TTT TTg AgT CCC CTT AgT ATT TAT T 3'; U3 primer 5' Agg TAT TgT CCA Agg TTT CTC C 3', both at 0.3 mM. RT was performed at 50°C for 30 min directly followed by 94° C 2 min; 94° C 30 sec, 68° C 30 sec, -1.3° C each cycle, 68° C 45 sec for a total of 10 cycles; 94° C 30 sec, 55° C 30 sec, 68° C 45 sec for a total of 25 cycles. The amplificd material (487 bp) was subjected to agarose gel electrophoresis followed by alkaline transfer and hybridisation with probes generated from the IDDMK$_{1,2}$22 U3-R-region. Genomic PCR. 100 ng of genomic DNA was subjected to PCR. Reactions contained at a final concentration: dNTPs at 200 mM; 1.5 mM $MgCl_2$; 2.6 U of Taq-Pwo (Boehringer Mannheim); U3-primer 5' Agg TAT TgT CCA Agg TTT CTC C 3'; R-primers either 5' CTT TAC AAA gCA gTA TTg CTg C 3, or 5' gTA AAg gAT CAA gTg CTg TgC 3' at 300 nM. The amplified products were 300 and 395 bp in size, respectively. The cycling profile was as follows: 94° C 2 min; 94° C 15 sec, 68° C 30 sec, -1.3° C each cycle, 72° C 45 sec for a total of 10 cycles; 94° C 15 sec, 55° C 30 sec, 72° C 45 sec for a total of 25 cycles.

Sequence alignment and phylogenetic trees

Sequences were aligned with CLUSTAL W (Thompson et al., 1994). Alignments were checked and manually corrected with the SEA VIEW multiple sequence alignment editor (Galtier et al., 1996). Phylogenetic trees were computed from multiple alignments using the ``neighbour joining'' method (Saitou and Nei, 1987).

Expression

Constructs. pPOL-ENV-U3: a SacI-NotI fragment derived from 11 IDDMK$_{1,2}$22 clones was ligated with 1) a BamHI-SacI adapter containing a consensus SD and 2) with a NotI-XbaI adapter and 3) was subcloned into BamHI-XbaI digested p1DR2-arms, selected for by two rounds of screening and plasmids rescued. At least five independent clones were used for transfections. pPOL: pPOL-ENV-U3 was digested with KpnI-NotI, blunted and religated. pPOL-ENV/TR: a stimulatory clone was digested with XbaI and religated. pCI-ENV/TR: 1 ng of pPOL-ENV-U3 was amplified with the primers 5' gAC TAA gCT TAA gAA CCC ATC AgA gAT gC 3' and 5' AgA CTg gAT CCg TTA AgT CgC TAT CgA CAg C 3'. The amplified products were subcloned into pCI-neo (Promega).

Cells and cell lines. Monocytic cell lines: THP1, U937. B-lymphoblastoid cell lines: Raji, BOLETH, SCHU and WT 51. T cells of molecularly MHC-typed blood donors were generated by positive selection with anti-CD3 coated immunomagnetic beads (Milan-Analytika).

Transfections. Transient transfectants were used for functional assays 48 hours after transfection; stable transfectants were selected for 2 weeks in progressive concentration of Hygromycin B to a final concentration of 250 mg/ml for lymphoblastoid lines, and 50 mg/ml for monocytic cell lines.

Functional assays. Transfectants were treated with Mitomycin C (Calbiochem) at 100 mg/ml per 10^7 cells for 1 hour at 37° C and washed extensively. Proliferation assays. 10^5 CD3-beads-selected, MHC compatible T cells or Ficoll-Paque-isolated allogeneic PBL were cultured with transfectants at stimulator: responder ratios of 1:1; 1:3 and 1:10 for 48 and 72 hours in 96 round-bottom wells at 37° C. ^{3}H-Thymidine was then added at 1mCi/well and incorporation measured after 18 hours incubation at 37° C. FACS analysis and antibodies used were as described; after 3 days of specific stimulation, at T: non-T ratios of 1:1 for syngeneic, and 10:3 for allogeneic stimulations, the T cells were further expanded in 20 U/ml recombinant IL-2 for 6 days before flow cytometric analysis (Conrad et al., 1994).

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CLAIMS

1. Process for the diagnosis [illegible] human autoimmune disease, including [illegible] diagnosis, said human autoimmune [illegible] being associated with human retrovirus having superantigen (SAg) activity, comprising specifically [illegible] in a biological sample of human origin at least [illegible] the following :

I- the mRNA of an expressed human retrovirus having Superantigen (SAg) activity, or [illegible] of such expressed retroviral mRNA, said retrovirus being associated with a given autoimmune disease, or

II- protein expressed by said retrovirus, or

III- antibodies specific to the protein [illegible] by said retrovirus, or

IV- SAg activity specifically associated with the autoimmune disease.

2. Process according to claim 1 wherein the retrovirus having superantigen activity is human endogenous retrovirus (HERV).

3. Process according to claim 1 or 2 wherein the expressed retroviral mRNA is specifically detected by nucleic acid amplification using primers at least one of which is specific for the poly(A) signals present in the 3' R-poly(A) sequences at the 3' extremity of the retrovirus.

4. Process according to claim 1 or 2 wherein the protein expressed by the retrovirus is detected using antibodies specific for the said retrovirus protein.

5. Process according to claim 1 wherein the antibodies specific to retroviral protein are detected by use of the corresponding retroviral proteins or fragments thereof.

6. Process according to claim 1 wherein SAg activity specifically associated with said autoimmune disease is detected by contacting a biological fluid containing MHC Class II^+ cells with cells bearing one or more variable (V)-β T-cell receptor chains and detecting preferential proliferation of the V subset characteristic of said autoimmune disease.

7. Process according to claim 1 wherein the autoimmune disease is type I diabetes and the associated retrovirus having SAg activity ($IDDMK_{1,2}$ 22 comprising the 5' long terminal repeat shown in Figure 7A, the 3' short terminal repeat shown in Figure 7B, or the env encoding sequences shown in Figure 7C, Figure 7D or Figure 7E, or variants thereof presenting approximately 90% sequence identity.

8. Process according to claim 7 wherein the expressed retroviral RNA is specifically detected by nucleic acid amplification using primers, one of which is specific for the poly(A) signals present in the 3' R-poly(A) sequences at the 3' extremity of $IDDMK_{1,2}$ 22.

9. Process according to claim 8 wherein the poly(A) specific primer is

5' TTTTTGAGTCCCCTTAGTATTTATT 3'

10. Process according to claim 7 wherein protein expressed by $IDDMK_{1,2}$ 22 is detected, said protein being either the protein encoded by the N-terminal moiety of

the env coding region of IDDMK$_{1,2}$22 as illustrated in Figure 7D or 7G, or the protein encoded by the pol coding region, as illustrated in Figure [illegible], or a protein having at least 90% homology with the illustrated protein, or a fragment of said proteins having at least 6 amino-acids.

11. Process according to claim [illegible] wherein antibodies specific for env or pol proteins expressed by IDDMK$_{1,2}$22 are detected using the env or pol proteins illustrated in Figure 7D, 7G or [illegible] or a protein having at least 90% homology with the illustrated protein, or a fragment of said proteins having at least 6 amino-acids.

12. Human retrovirus having [illegible] antigen activity, and being associated with human autoimmune disease.

13. Retrovirus according to claim [illegible] obtainable from RNA prepared from a biological sample of human origin, by carrying out the following steps :

i) isolation of the 5' R-U5 ends of unprocessed putative retroviral genomes using [illegible] acid amplification, the 3' primer being complementary to known « primer binding sites » (pbs) ;

ii) isolation of the 3' R [illegible] ends corresponding to the 5' R-U5 ends, by using primers specific for the R regions isolated in step i) ;

iii) amplification of the conserved [illegible] H region within the pol gene by using degenerate primers corresponding to the conserved region ;

iv) amplification of the 5' [illegible] of the putative retroviral genome by using primers specific for the different U5 regions isolated in step i) in

conjunction with a primer specific for the 3' end of the central pol region isolated in step iii) ;

v) amplification of the 3' moiety of the putative retroviral genome using primers specific for the central pol region isolated in step iii) in conjunction with primers specific for the poly(A) sequences present in the 3' R-poly(A) sequences isolated in step ii) ;

vi) confirmation of the presence of an intact retroviral genome by amplification using primers specific for its predicted U5 and U3 regions.

14. Proviral DNA of a retrovirus according to claim 12 or 13.

15. Proviral DNA according to claim 14 obtainable from a biological sample of human origin by

i) obtaining retroviral RNA according to the method of claim 13, and further,

ii) generating a series of DNA probes from the retroviral RNA obtained in i);

iii) hybridising under stringent conditions, the probes on a genomic human DNA library

iv) isolation of the genomic sequences hybridising with the probes.

16. Nucleic acid molecule comprising fragments of the retroviral RNA or DNA according to any one of claims 12 to 15, said fragment having a length of at least 15 nucleotides and preferably at least 30 nucleotides.

17. Nucleic acid molecule according to claim 16, encoding SAg activity of the retrovirus.

18. Nucleic acid molecule according to claim 17 derived from an endogenous human retrovirus and containing at least one internal stop codon.

19. Nucleic acid molecule comprising a sequence complementary to the nucleic acid molecules of any one of claims 12 to 18.

20. Nucleic acid molecule according to claim 19 comprising a ribozyme or antisense molecule to a human retrovirus having SAg activity to a proviral DNA of said retrovirus or a fragment thereof.

21. Nucleic acid molecule capable of hybridizing in stringent conditions, with the nucleic acid molecules of any one of claims 12 to 20.

22. Vector comprising nucleic acid molecules of any one of claims 12 to 21.

23. Nucleic acid molecule comprising at least one of the sequences illustrated in Figures 7A, 7B, 7D, 7E, or a nucleic acid sequence encoding the SAg protein shown in Figure 7H, or a sequence exhibiting at least 90% homology with any of these sequences, or a fragment of any of these sequences having at least 20 nucleotides.

24. Nucleic acid molecule at least partially complementary to any of the sequences according to claim 23.

25. Nucleic acid molecule according to claim 23 comprising a ribozyme or antisense.

26. Nucleic acid molecule which is HERV IDDMK$_{1,2}$-22 comprising each of the sequences illustrated in Figures 7A, 7B, 7C, or sequences having at least 90% identity with these sequences, having a size of approximately 8.5 kb, having SAg activity encoded within the **env** region illustrated in Figure 7D or 7E, said SAg activity being specific for Vβ7 - TCR chains.

27. Protein expressed by a human retrovirus having SAg activity and being associated with human autoimmune disease, or fragment of said protein having at least 6 amino acids.

28. Protein according to claim 27 wherein the protein or fragment thereof has SAg activity.

29. Protein according to claim 28 wherein said proteing having SAg activity results from a premature translational stop, and possibly also from a translational frame shift.

30. Protein according to any one of claims 27 to 29 obtainable by introducing proviral DNA of claim 15 or fragments thereof into a eukaryotic cell under conditions allowing the DNA to be expressed, and recovering said protein.

31. Protein according to any one of claims 27 to 29 comprising the amino acid sequence shown in Figure 7D, Figure 7F, Figure 7G, Figure 7H, or an amino acid sequence having at least 80% and preferably at least 90 % homology with the illustrated sequences, or a fragment of said sequence having at least 6 amino acids.

32. Antibodies capable of specifically recognising a protein according to any one of claims 27 to 31.

33. Antibodies according to claim 32 which are monoclonal.

34. Antibodies according to claim 32 or 33 which specifically recognise a HERV protein having SAg activity and which have the capacity to block SAg activity.

35. Cell-line transfected with and expressing a human retrovirus or a nucleic acid molecule according to any one of claims 12 to 26.

36. Non-human cells transformed with and expressing a human retrovirus or a nucleic acid molecule according to any one of claims 12 to 26.

37. Cell-line or cells according to claim 35 or 36, said cell-lines or cells being MHC Class II^+ and expressing a protein having SAg activity.

38. Process for identifying substances capable of binding to retroviral protein having SAg activity involved in autoimmune disease, comprising contacting the substance under test, optionally labelled with detectable marker, with the retroviral protein having SAg activity, and detecting binding.

39. Process for identifying substances capable of blocking SAg activity of a retrovirus associated with autoimmune disease, comprising introducing the substance under test into an assay system comprising i) MHC Class II^+ cells expressing retroviral protein

having SAg activity and ii) cells bearing Vβ-T cell receptor chains of the family or families specifically stimulated by the HERV SAg expressed by the MHC Class II^+ cells, and determining the capacity of the substance under test to diminish or block Vβ-specific stimulation by the retroviral SAg.

40. Process according to claim 39 wherein the cells bearing Vβ-T cell receptor chains are T-cell hybridoma and Vβ-specific stimulation is determined by measurement of IL-2 release.

41. Process according to claim 39 or 40, comprising an additional preliminary screening step for selecting substances capable of binding to retroviral protein having SAg activity, said screening step being according to claim 38.

42. Process for identifying substances capable of blocking transcription or translation of human retroviral SAg-encoding nucleic acid sequences, said SAg being associated with a human autoimmune disease, comprising :

i) contacting the substance under test with cells expressing retroviral protein having SAg activity, and

ii) detecting loss of SAg protein expression using SAg protein markers such as specific, labelled anti-SAg antibodies.

43. Process according to claim 42 the cells expressing HERV protein having SAg activity are MHC Class II^+ cells, and the process further comprises detection of loss of SAg activity by the process of claim 39.

44. Kit for screening substances capable of blocking SAg activity of a retrovirus associated with an autoimmune disease, or of blocking transcription or translation of the retroviral SAg protein, comprising :
- MHC Class II^+ cells transformed with and expressing retroviral SAg ;
- cells bearing Vβ T-cell receptor chains of the family or families specifically stimulated by the HERV SAg ;
- means to detect specific Vβ stimulation by HERV SAg ;
- optionally, labelled antibodies specifically binding to the retroviral SAg.

45. Protein derived from a retroviral SAg according to claim 28 wherein the protein is modified so as to be devoid of SAg activity and is capable of generating a immune response against SAg, involving either antibodies and/or T-cell responses.

46. Protein according to claim 45 wherein the modification consists of denaturation, or of a truncation, or of a deletion, insertion or replacement mutation of the SAg protein.

47. Protein according to claim 45 or 46 for use as a prophylactic or therapeutic vaccine against autoimmune disease associated with retroviral SAg.

48. Vaccine comprising an immunogenically effective amount of a protein according to claim 45 or 46 in association with a pharmaceutically acceptable carrier and optionally adjuvant.

49. Nucleic acid molecule encoding human retroviral SAg according to claim 17 or a modified form of said molecule for use as a prophylactic or

therapeutic DNA vaccine against autoimmune disease associated with the retroviral SAg.

50. Substances identifiable by the process according to any one of claims 38 to 43 for use in therapy of autoimmune disease associated with the HERV SAg.

51. Use of substances capable of inhibiting retroviral function for the preparation of a medicament for use in therapy of autoimmune disease associated with retroviral SAg.

52. Use according to claim 51 wherein the substance capable of inhibiting retroviral function is A.Z.T.

53. Use of substances capable of inhibiting retroviral SAg function for the preparation of a medicament for use in therapy of autoimmune disease associated with retroviral SAg.

54. Process for detecting human autoimmune disease associated with expression of human retrovirus Superantigen (SAg), said process comprising at least one of the following steps :

i) detecting the presence of any expressed retrovirus in a biological sample of human origin ;

ii) detecting the presence of SAg activity in a biological sample of human origin containing MHC Class II^+ cells.

55. Process according to claim 54 wherein the expressed retrovirus is detected by detection of reverse transcriptase activity.

56. Process according to claim 55 wherein the expressed retrovirus is detected by carrying out nucleic acid amplification reaction on RNA prepared from the biological sample, using as 3' primer a sequence complementary to known retroviral « primer binding sites » (pbs), and as 5' primer a non-specific anchor sequence.

57. Process according to claim 54 wherein the presence of SAg activity is detected by contacting the biological sample containing MHC Class II^+ cells with cells bearing one or more variable (V)-β T-cell receptor (TCR) chains and detecting preferential proliferation of a Vβ subset.

58. Process according to claim 57 wherein the cells bearing T-cell receptors are T-cell hybridoma bearing defined human Vβ domains.

59. Process for detecting SAg activity of an expressed human retrovirus associated with human autoimmune disease or of a portion of said retrovirus comprising :

i) transfecting expressed retroviral DNA or portions thereof into MHC Class II^+ antigen presenting cells under conditions in which the DNA is expressed,

ii) contacting the transfectants with cells bearing one or more defined (V)-β T-cell receptor chains, and

iii) determining whether the transfectant is capable of inducing preferential proliferation of a Vβ subset, the capacity to induce preferential proliferation being indicative of SAg activity within the transfected DNA or portion thereof.

60. Process for isolating and characterising a human retrovirus, particularly a human endogenous retrovirus (HERV), said retrovirus having SAg activity and being involved in human autoimmune disease, comprising the following steps :

i) isolation of the 5' R-U5 ends of expressed putative retroviral genomes using nucleic acid amplification, the 3' primer being complementary to known « primer binding sites » (pbs) ;

ii) isolation of the 3' R-poly(A) ends corresponding to the 5' R-U5 ends, by use of primers specific for the R regions isolated in step i) :

iii) amplification of the conserved RT-RNase H region within the pol gene by using degenerate primers corresponding to the conserved region ;

iv) amplification of the 5' moiety of the putative retroviral genome by using primers specific for the different U5 regions isolated in step i) in conjunction with a primer specific for the 3' end of the central pol region isolated in step iii) ;

v) amplification of the 3' moiety of the putative retroviral genome using primers specific for the central pol region isolated in step iii) in conjunction with primers specific for the poly(A) signals present in the 3' R-poly(A) sequences isolated in step ii) ;

vi) confirmation of the presence of an intact retroviral genome by amplification using primers specific for its predicted U5 and U3 regions.

61. Process according to claim 60 further comprising a step vii) of detecting SAg activity associated with the retrovirus, or portions thereof, said detection being carried out according to claim 59.

ASTRACT

The invention relates to a process for the diagnosis of a human autoimmune disease, including pre-symptomatic diagnosis, said human autoimmune disease being associated with human retrovirus having Superantigen (SAg) activity, comprising specifically detecting in a biological sample of human origin at least one of the following :

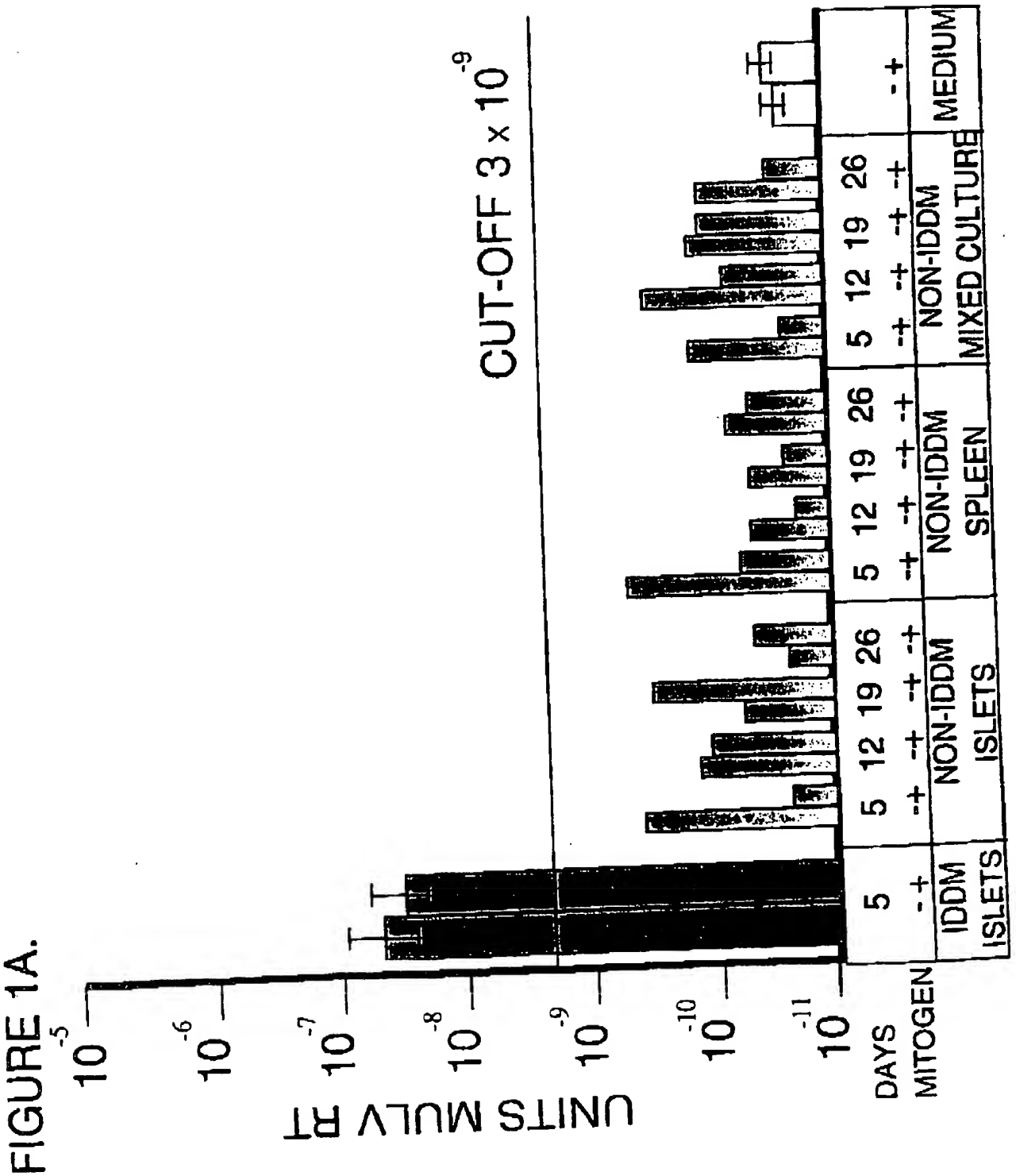
I- the mRNA of an expressed human retrovirus having Superantigen (SAg) activity, or fragments of such expressed retroviral mRNA, said retrovirus being associated with a given autoimmune disease, or

II- protein expressed by said retrovirus, or

III- antibodies specific to the proteins expressed by said retrovirus, or

IV- SAg activity specifically associated with the autoimmune disease.

FIGURE 1A.


UNITS MULV RT
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
10^{-11}
CUT-OFF 3×10^{-9}
DAYS
MITOGEN
5
- +
IDDM ISLETS
5 12 19 26
- + - + - + - +
NON-IDDM ISLETS
5 12 19 26
- + - + - + - +
NON-IDDM SPLEEN
5 12 19 26
- + - + - + - +
NON-IDDM MIXED CULTURE
- +
MEDIUM

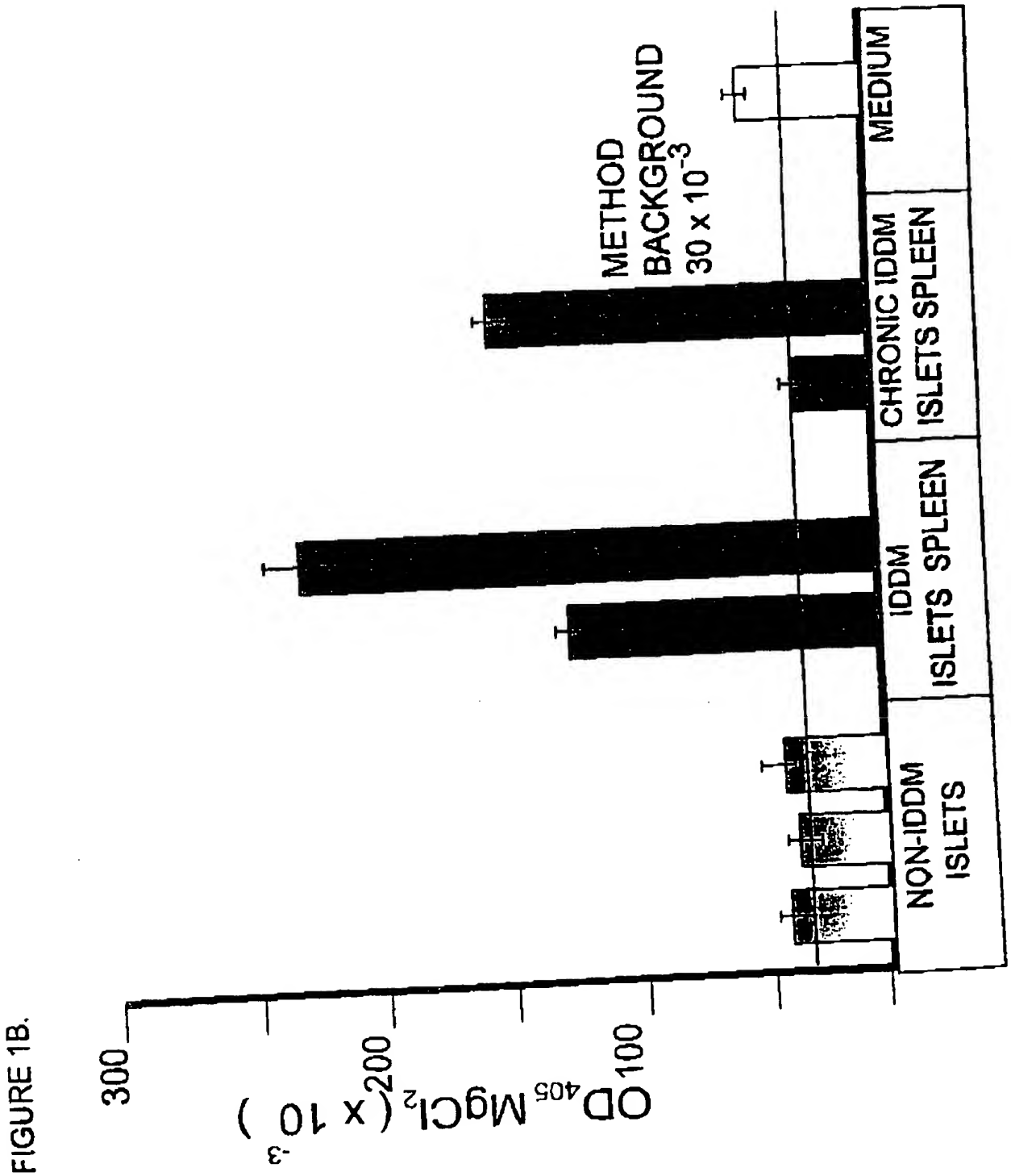

OD405 MgCl2 (x 10-3)
300
200
100
METHOD BACKGROUND 30 x 10-3
NON-IDDM ISLETS
IDDM ISLETS SPLEEN
CHRONIC IDDM ISLETS SPLEEN
MEDIUM

FIGURE 1B.

FIGURE 2A.

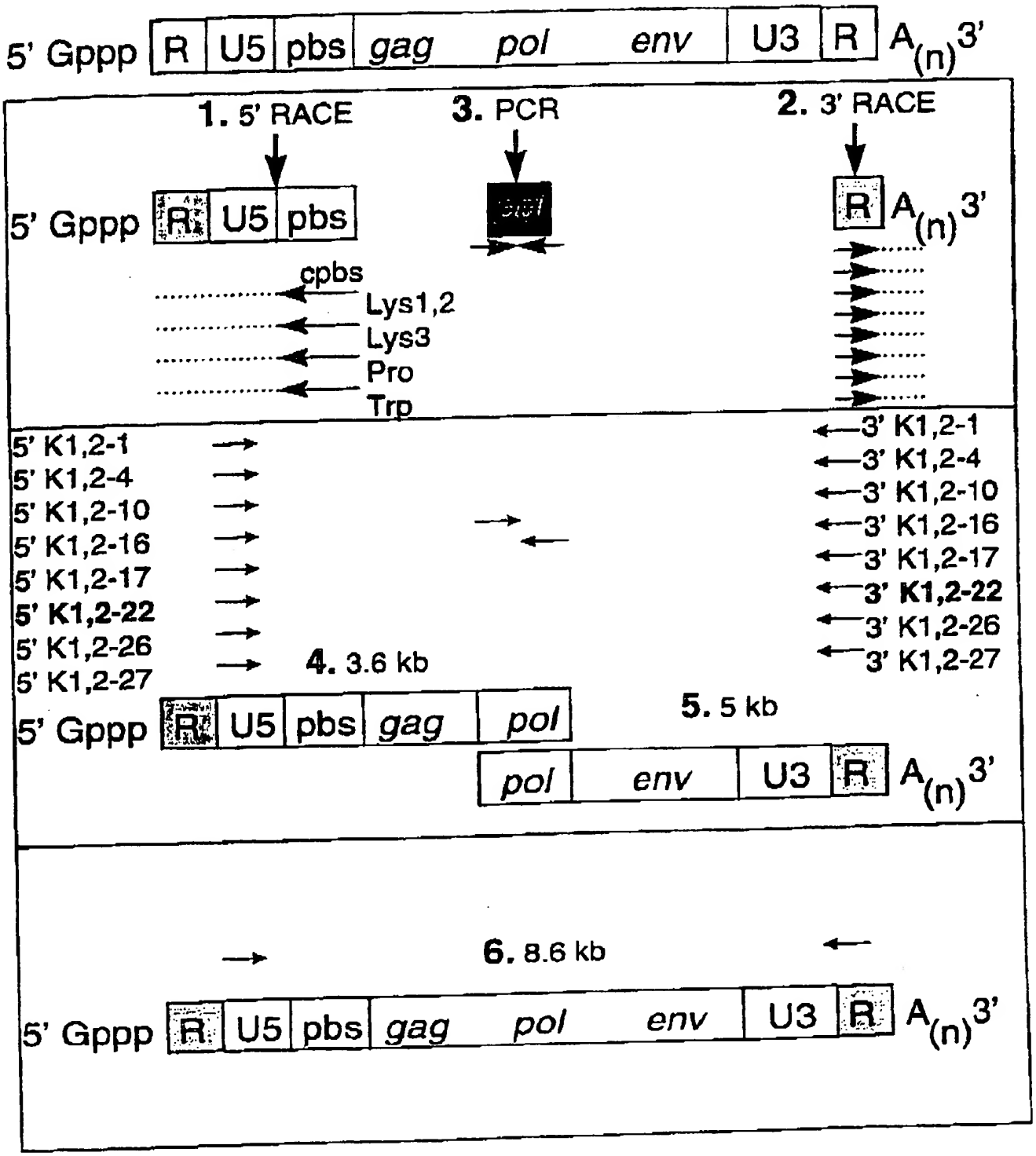

5' Gppp R U5 pbs gag pol env U3 R A(n)3'
1. 5' RACE
3. PCR
2. 3' RACE
5' Gppp R U5 pbs
R A(n)3'
cpbs
Lys1,2
Lys3
Pro
Trp
5' K1,2-1
5' K1,2-4
5' K1,2-10
5' K1,2-16
5' K1,2-17
5' K1,2-22
5' K1,2-26
5' K1,2-27
3' K1,2-1
3' K1,2-4
3' K1,2-10
3' K1,2-16
3' K1,2-17
3' K1,2-22
3' K1,2-26
3' K1,2-27
4. 3.6 kb
5' Gppp R U5 pbs gag pol
5. 5 kb
pol env U3 R A(n)3'
6. 8.6 kb
5' Gppp R U5 pbs gag pol env U3 R A(n)3'

FIGURE 2B

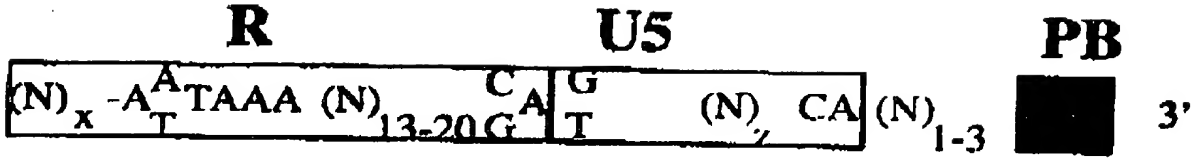


FIGURE 2C

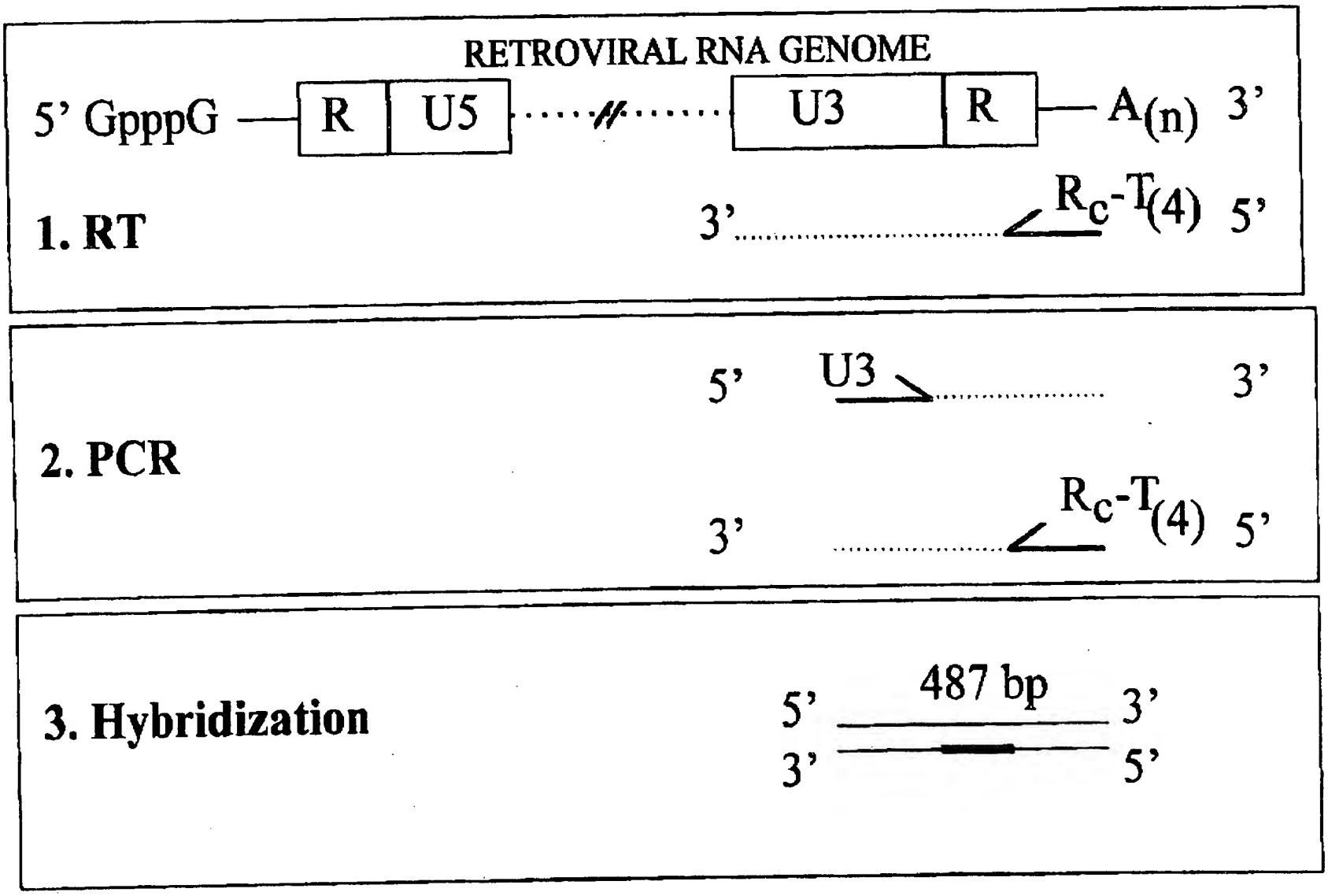


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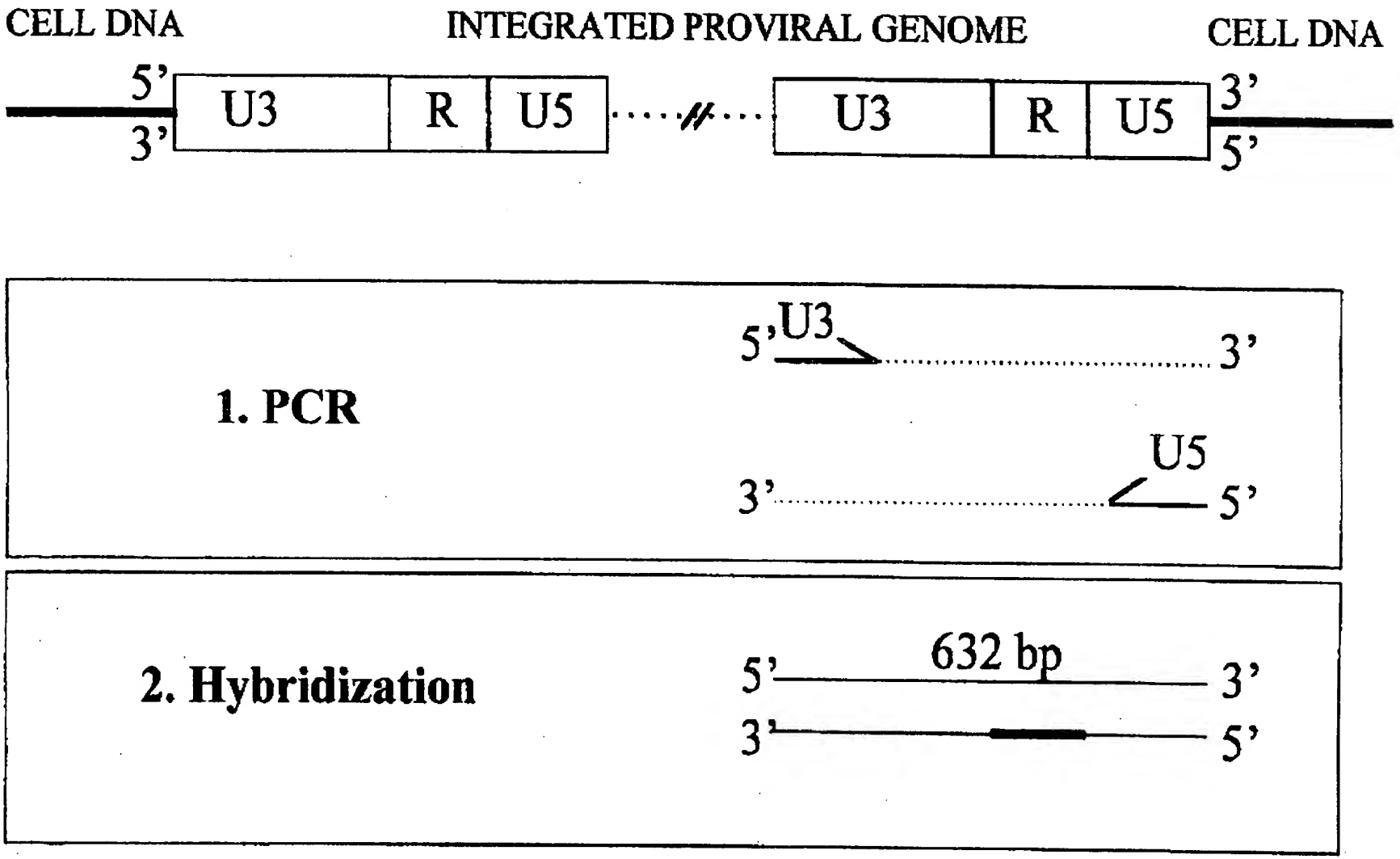
FIGURE 2D



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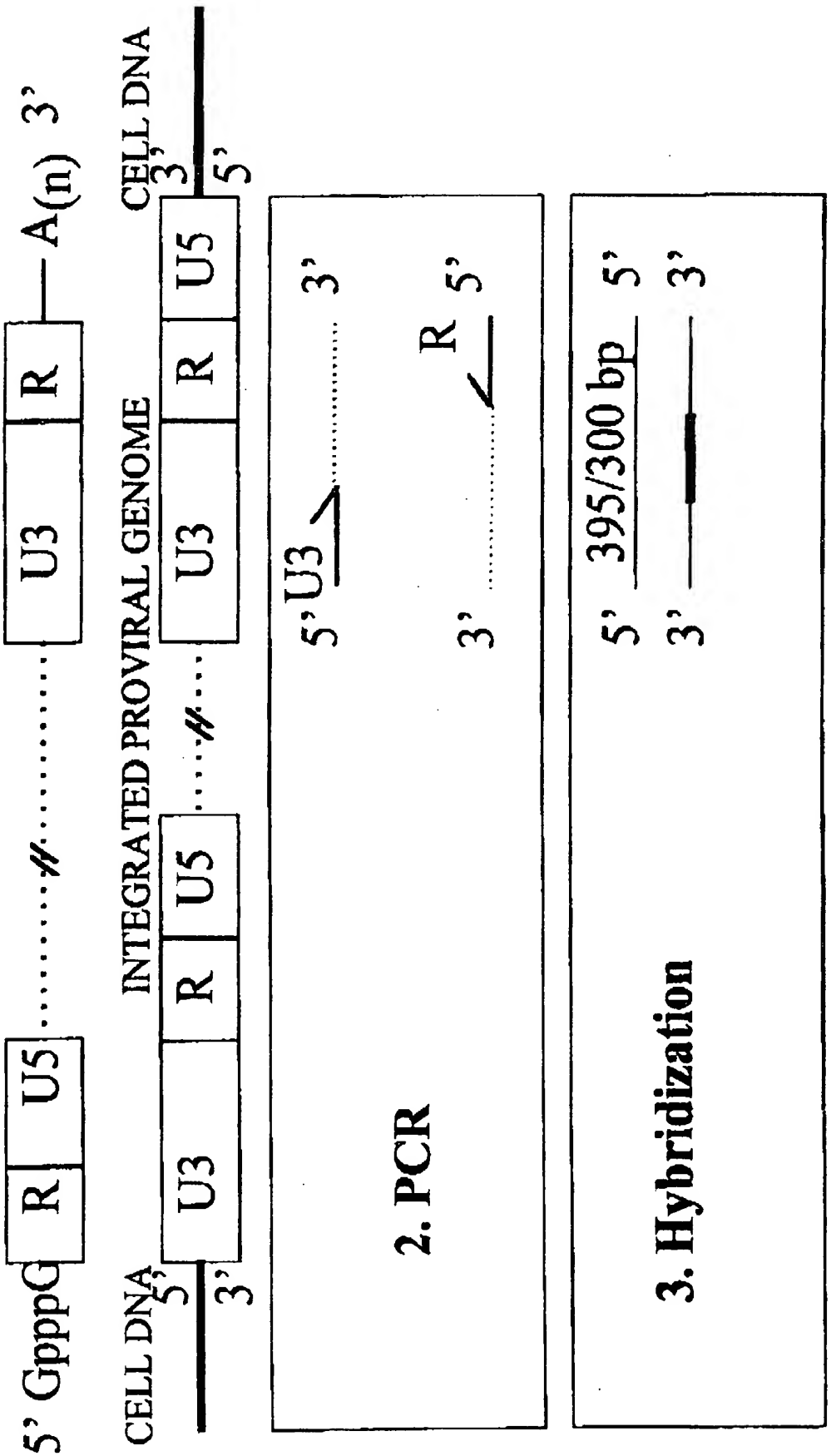
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5' GpppG
R
U5
U3
R
A(n) 3'
INTEGRATED PROVIRAL GENOME
CELL DNA
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3'
U3
R
U5
U3
R
U5
3'
5'
2. PCR
5' U3
3'
R 5'
3'
3. Hybridization
5' 395/300 bp 5'
3' 3'

FIGURE 2E

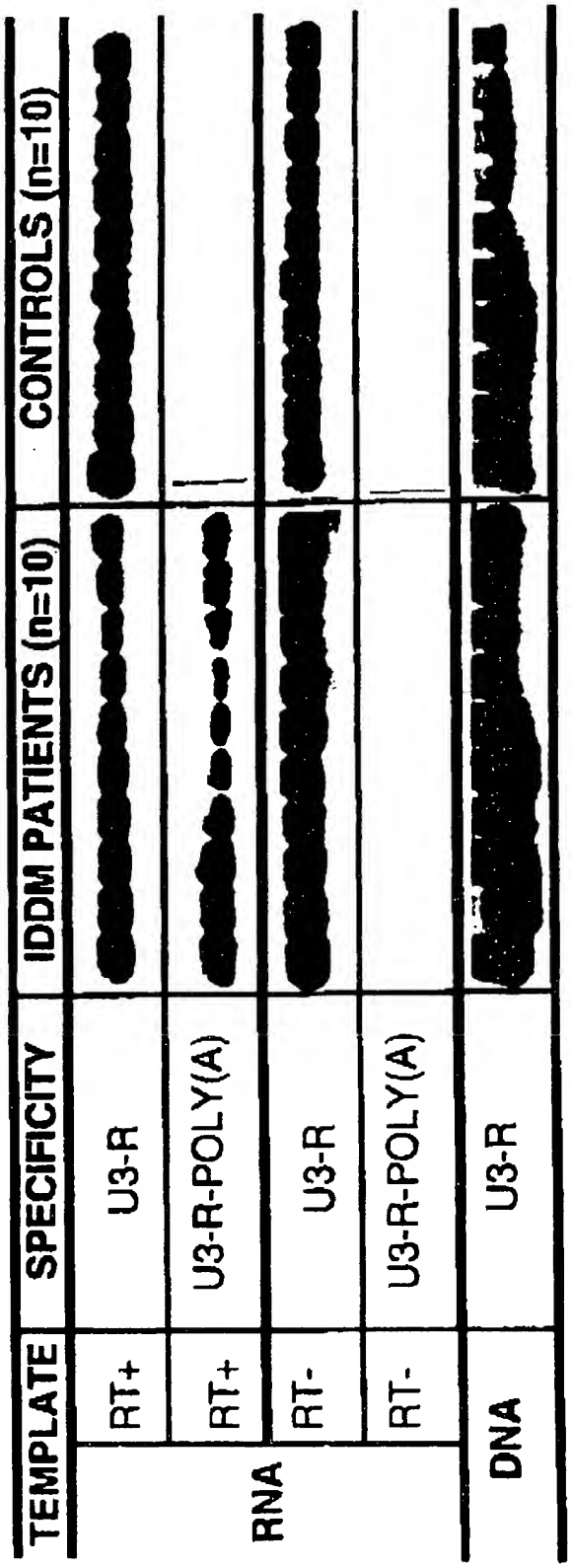
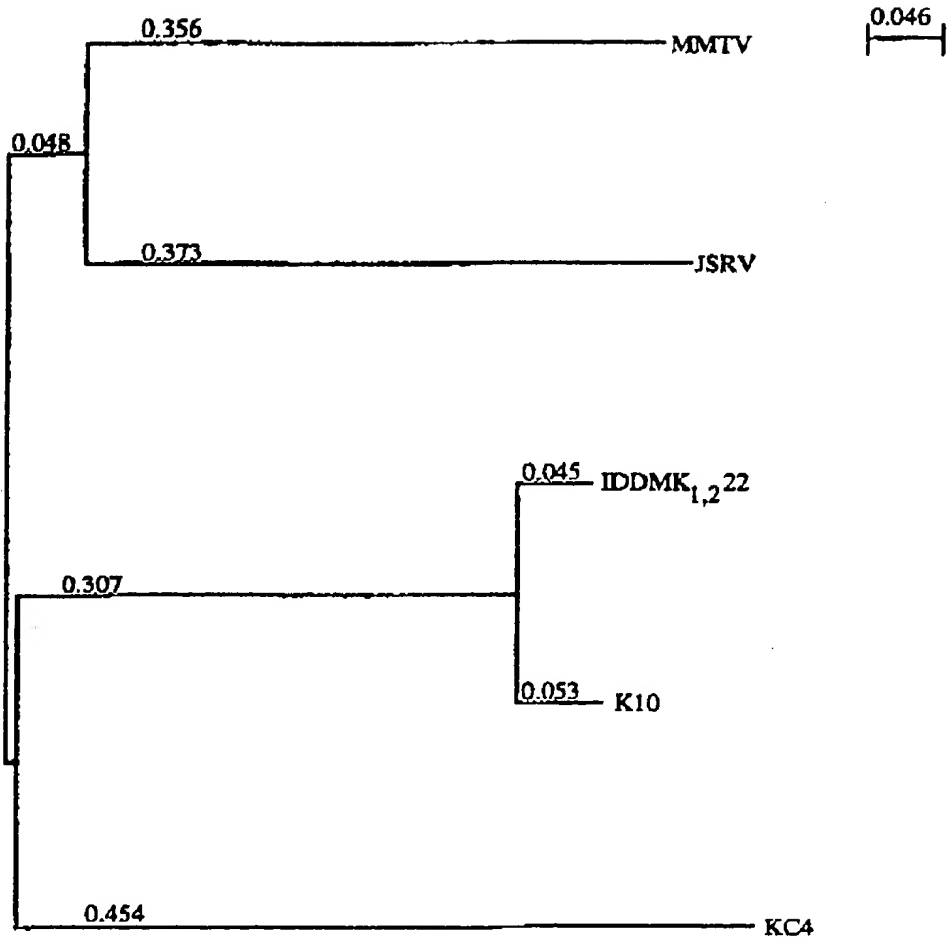

TEMPLATE
SPECIFICITY
IDDM PATIENTS (n=10)
CONTROLS (n=10)
RNA
RT+
U3-R
RT+
U3-R-POLY(A)
RT-
U3-R
RT-
U3-R-POLY(A)
DNA
U3-R

FIGURE 2F

FIGURE 3A.


0.356
MMTV
0.046
0.048
0.373
JSRV
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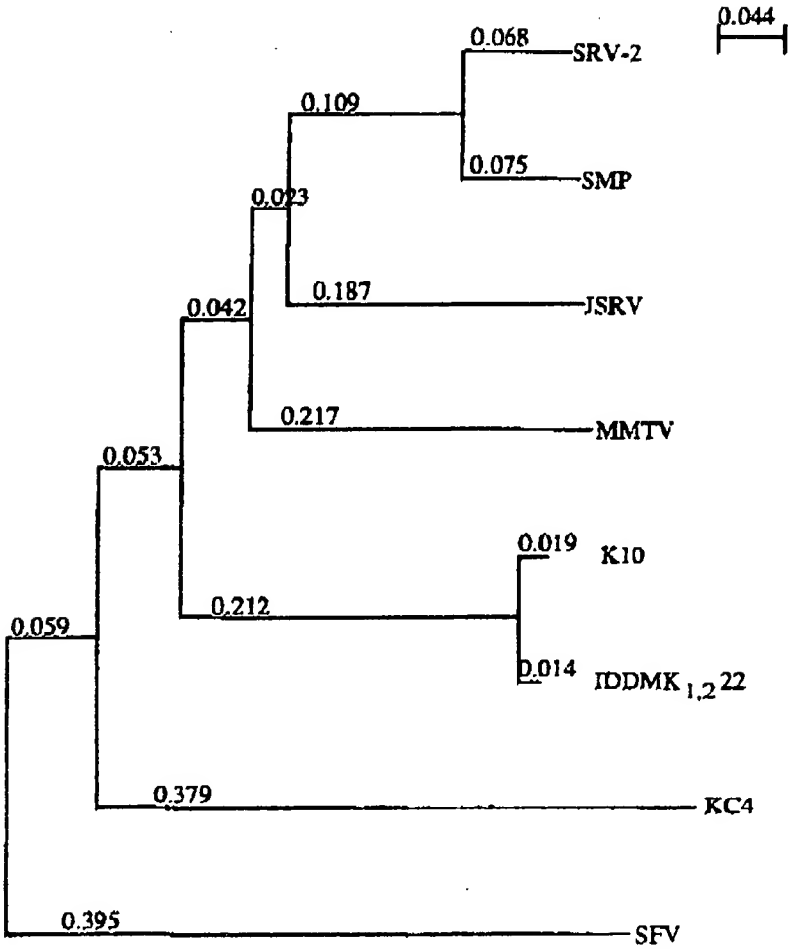
IDDMK1,222
0.307
0.053
K10
0.454
KC4

FIGURE 3B.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SRV-2
0.109
0.075
SMP
0.023
0.187
JSRV
0.042
0.217
MMTV
0.053
0.019
K10
0.212
0.059
0.014
IDDMK 1,2 22
0.379
KC4
0.395
SFV

FIGURE 3C.

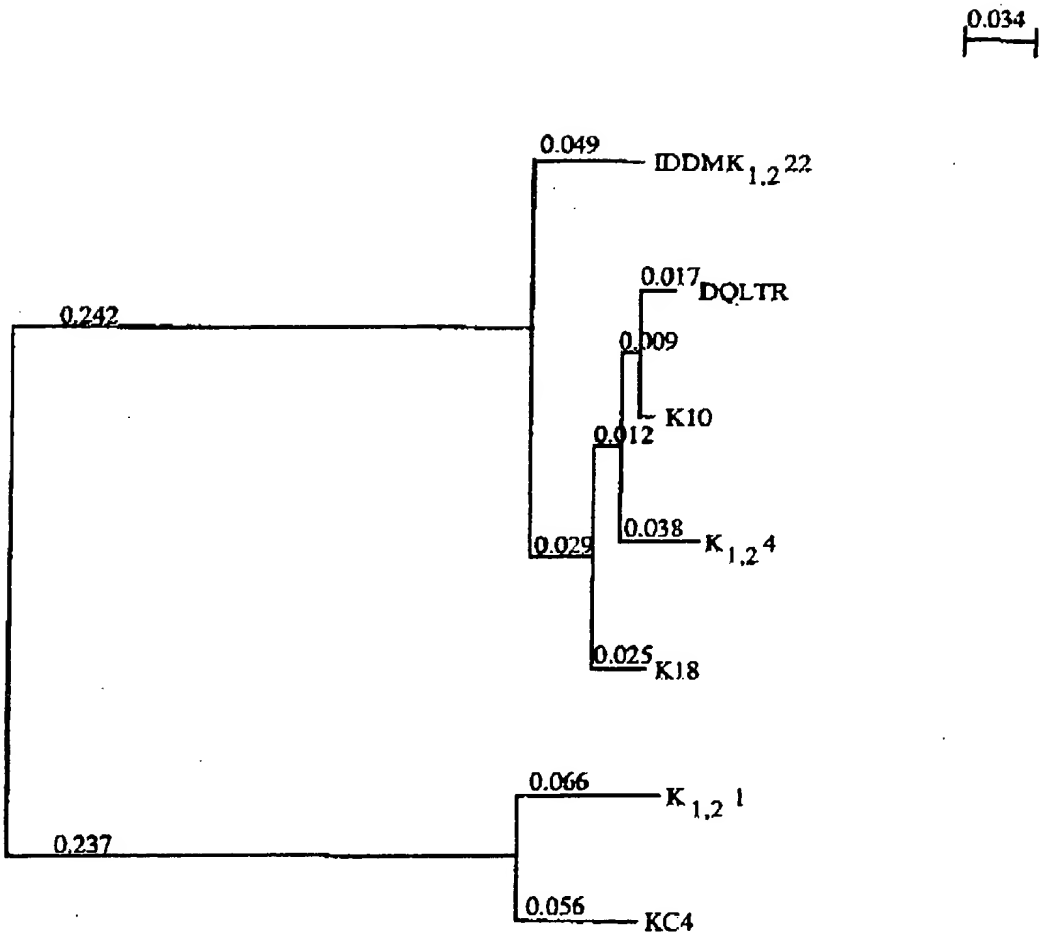
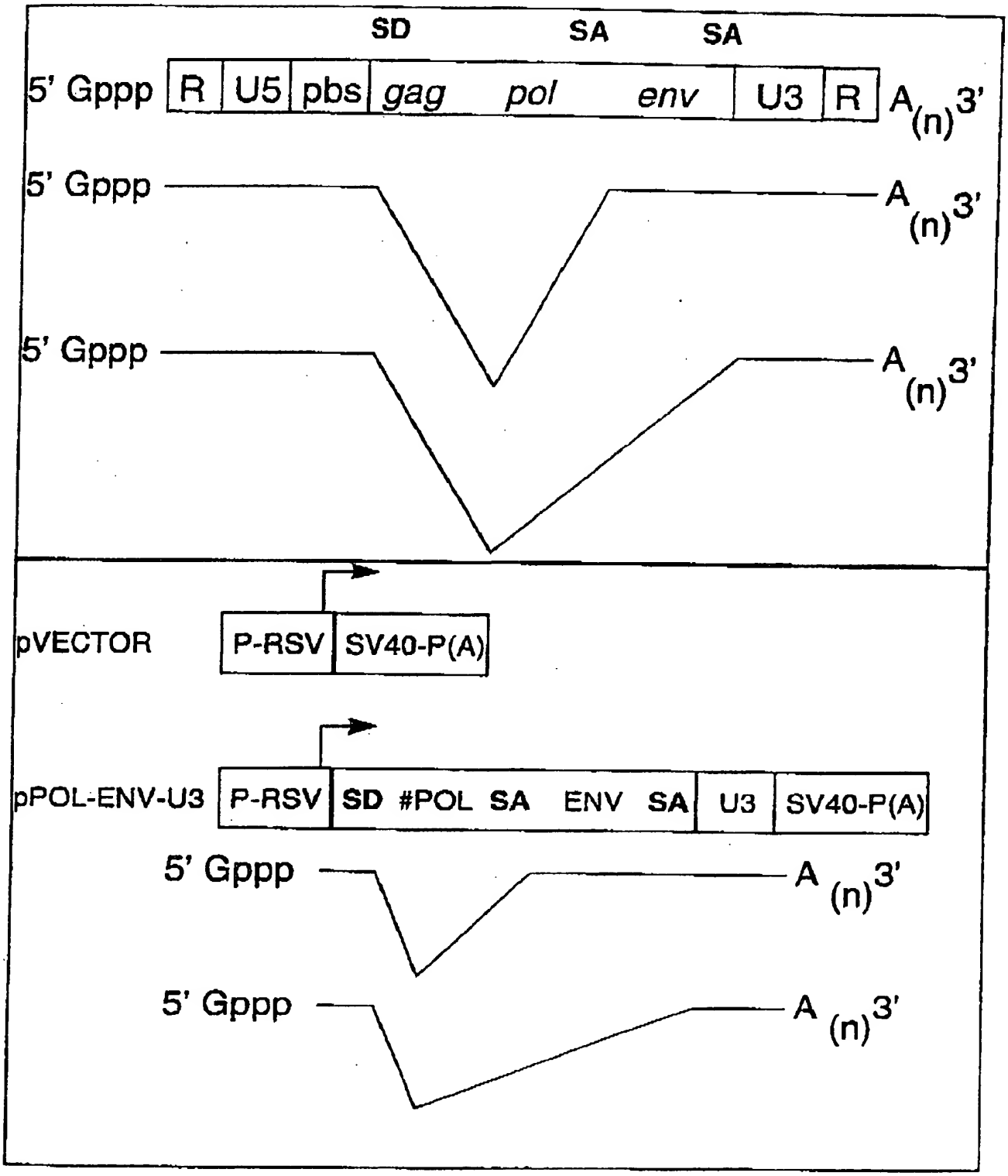

0.034
0.049
IDDMK1,222
0.017
DQLTR
0.242
0.009
K10
0.012
0.038
K1,24
0.029
0.025
K18
0.066
K1,21
0.237
0.056
KC4

FIGURE 4A.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SA
SA
5' Gppp
R
U5
pbs
gag
pol
env
U3
R
A(n)3'
5' Gppp
A(n)3'
5' Gppp
A(n)3'
pVECTOR
P-RSV
SV40-P(A)
pPOL-ENV-U3
P-RSV
SD #POL SA ENV SA
U3
SV40-P(A)
5' Gppp
A(n)3'
5' Gppp
A(n)3'

FIGURE 4B.

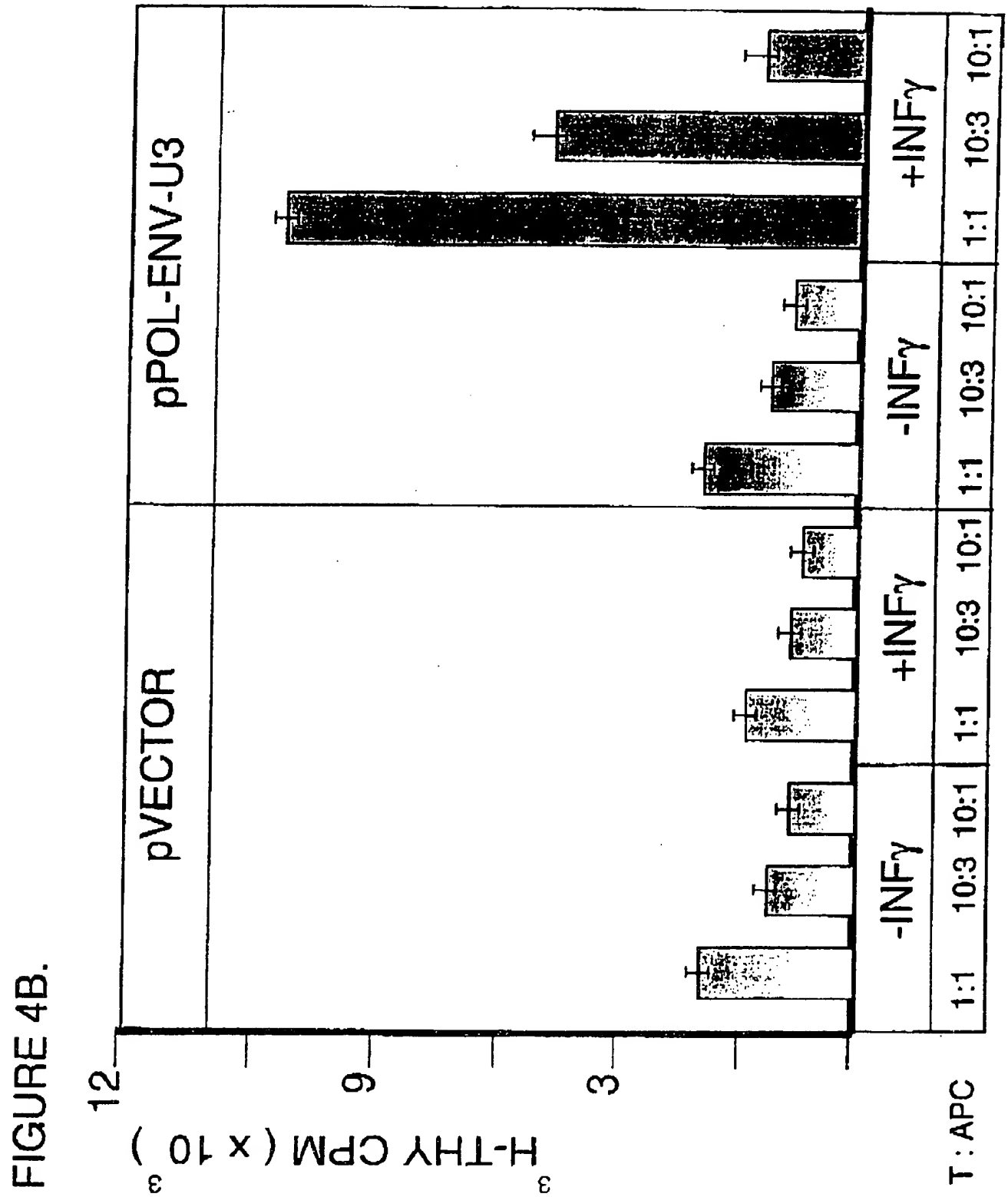
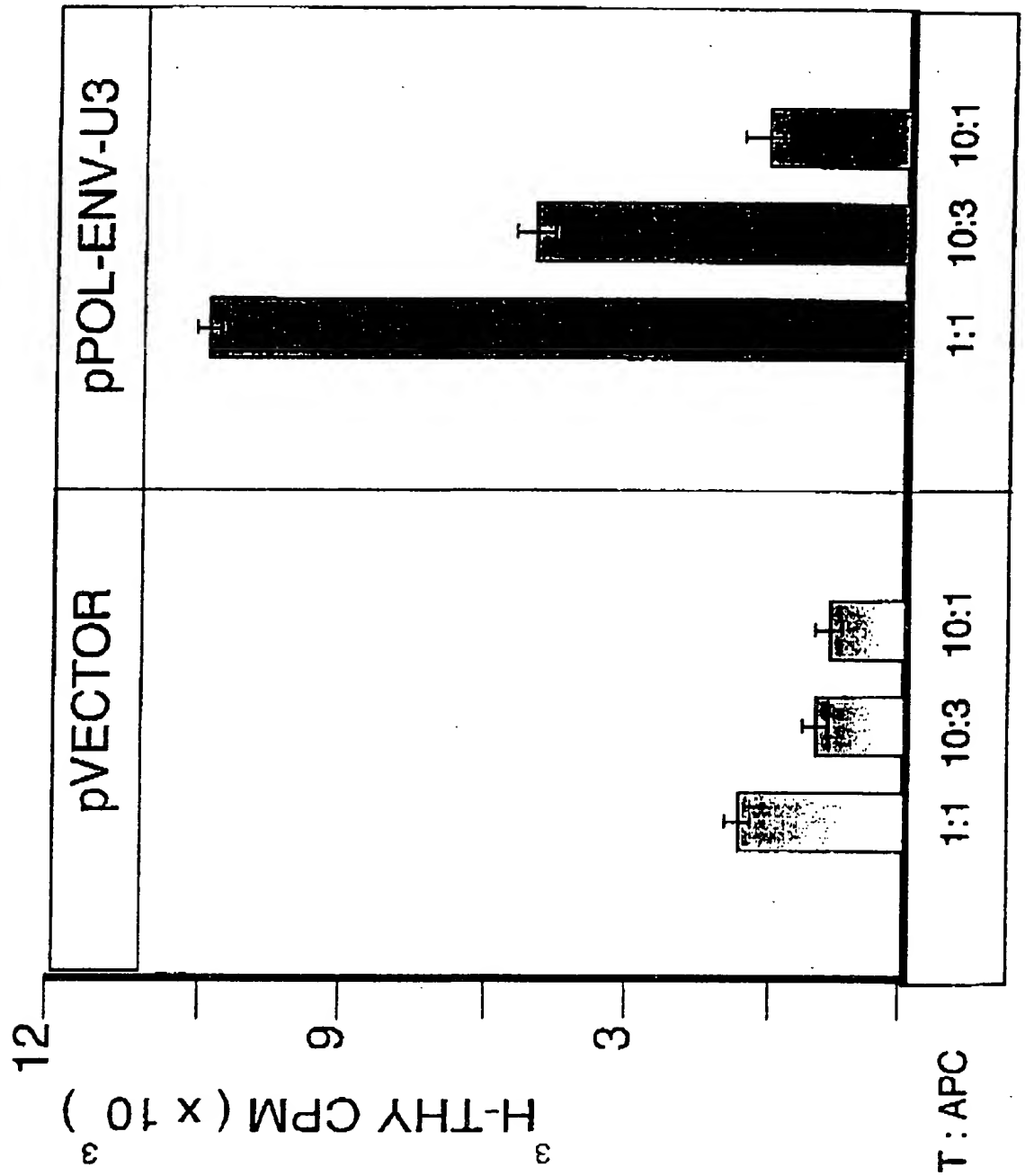

pVECTOR
pPOL-ENV-U3
-INFγ
+INFγ
-INFγ
+INFγ
1:1
10:3
10:1
1:1
10:3
10:1
1:1
10:3
10:1
1:1
10:3
10:1
12
9
6
3
H-THY CPM (x 10 3)
T : APC

FIGURE 4C.


pVECTOR
pPOL-ENV-U3
12
9
6
3
^{3}H-THY CPM (x 10 ^{3})
1:1
10:3
10:1
1:1
10:3
10:1
T : APC

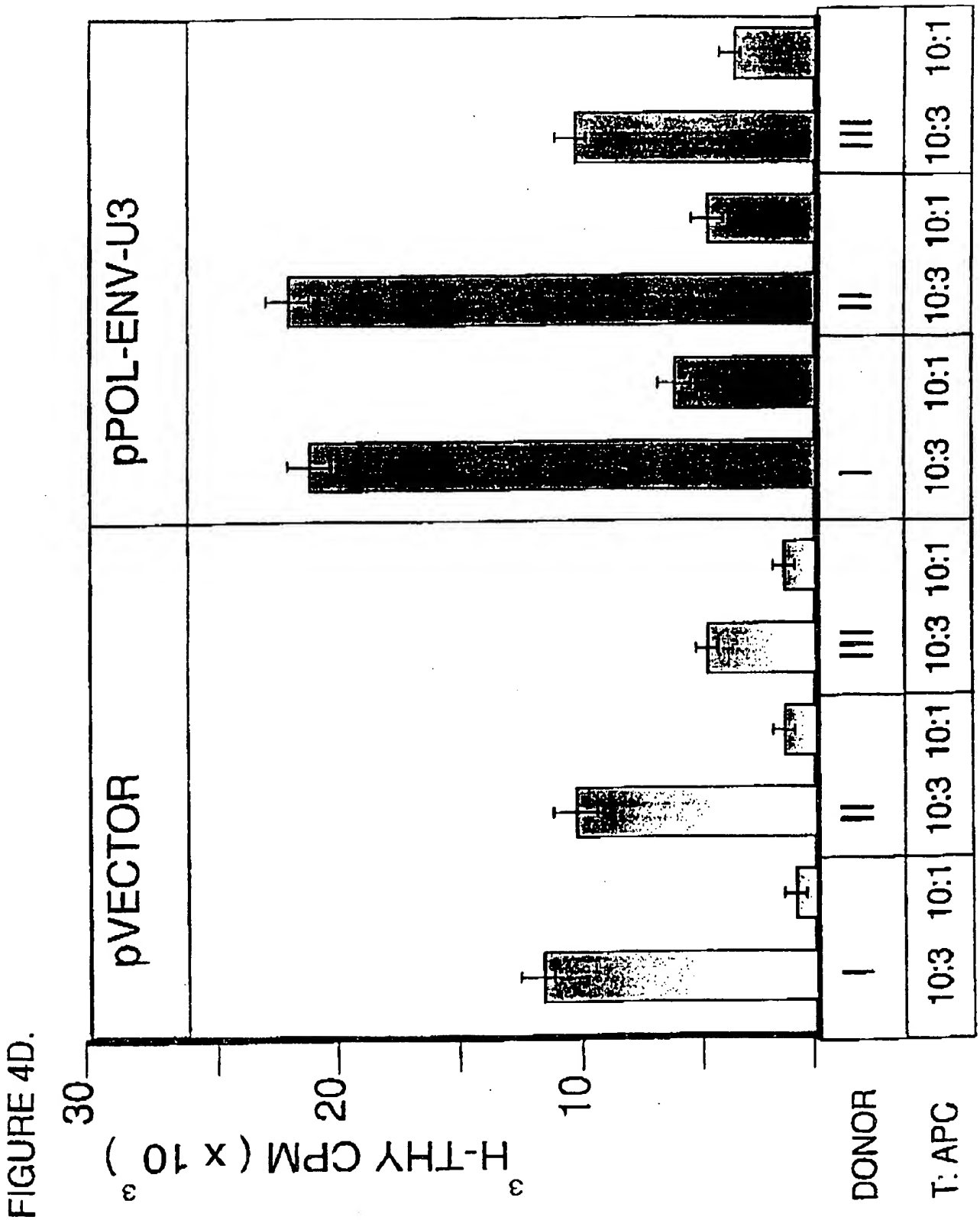

pVECTOR
pPOL-ENV-U3
^{3}H-THY CPM (x 10 ^{3})
30
20
10
DONOR
T: APC
I
II
III
10:3 10:1

FIGURE 4D.

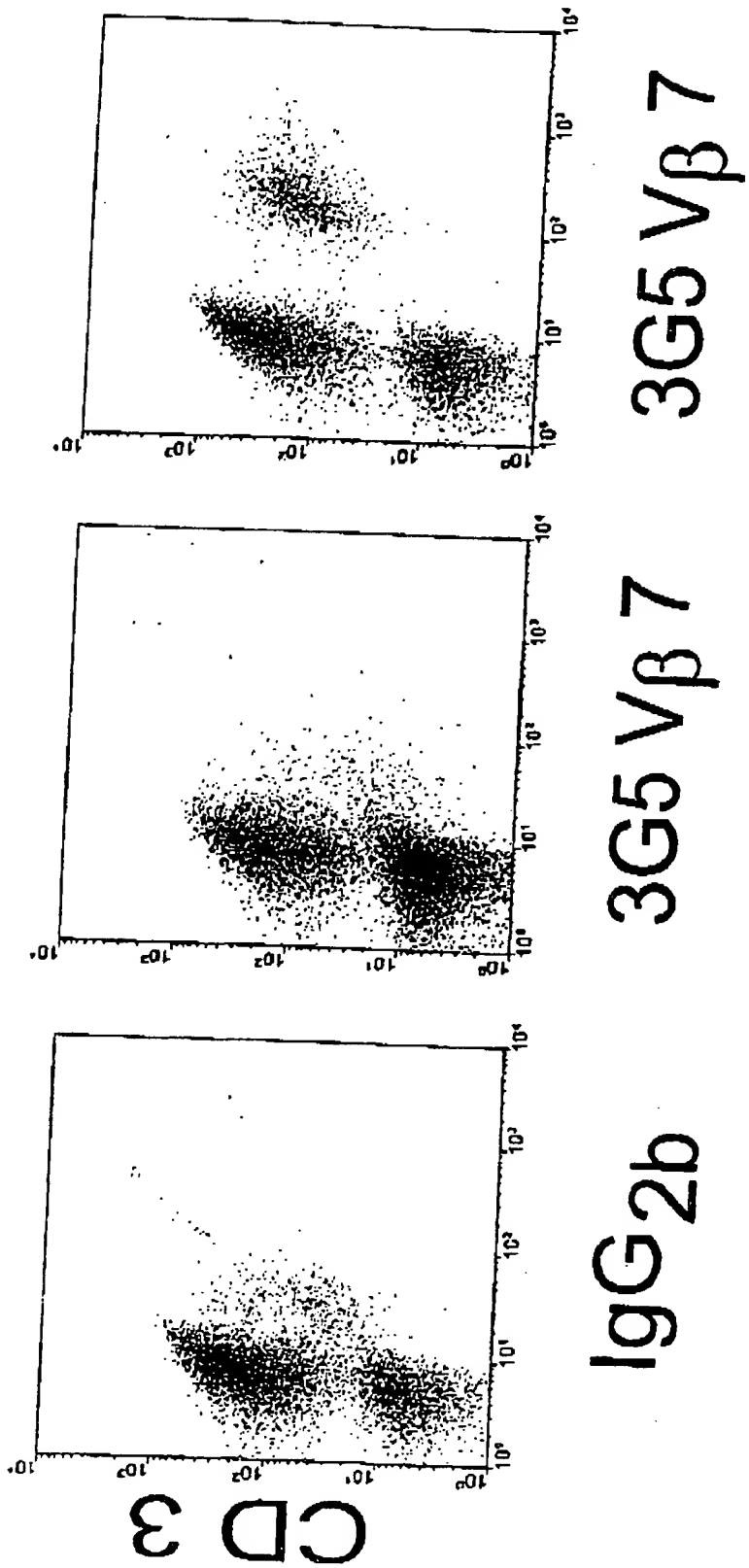
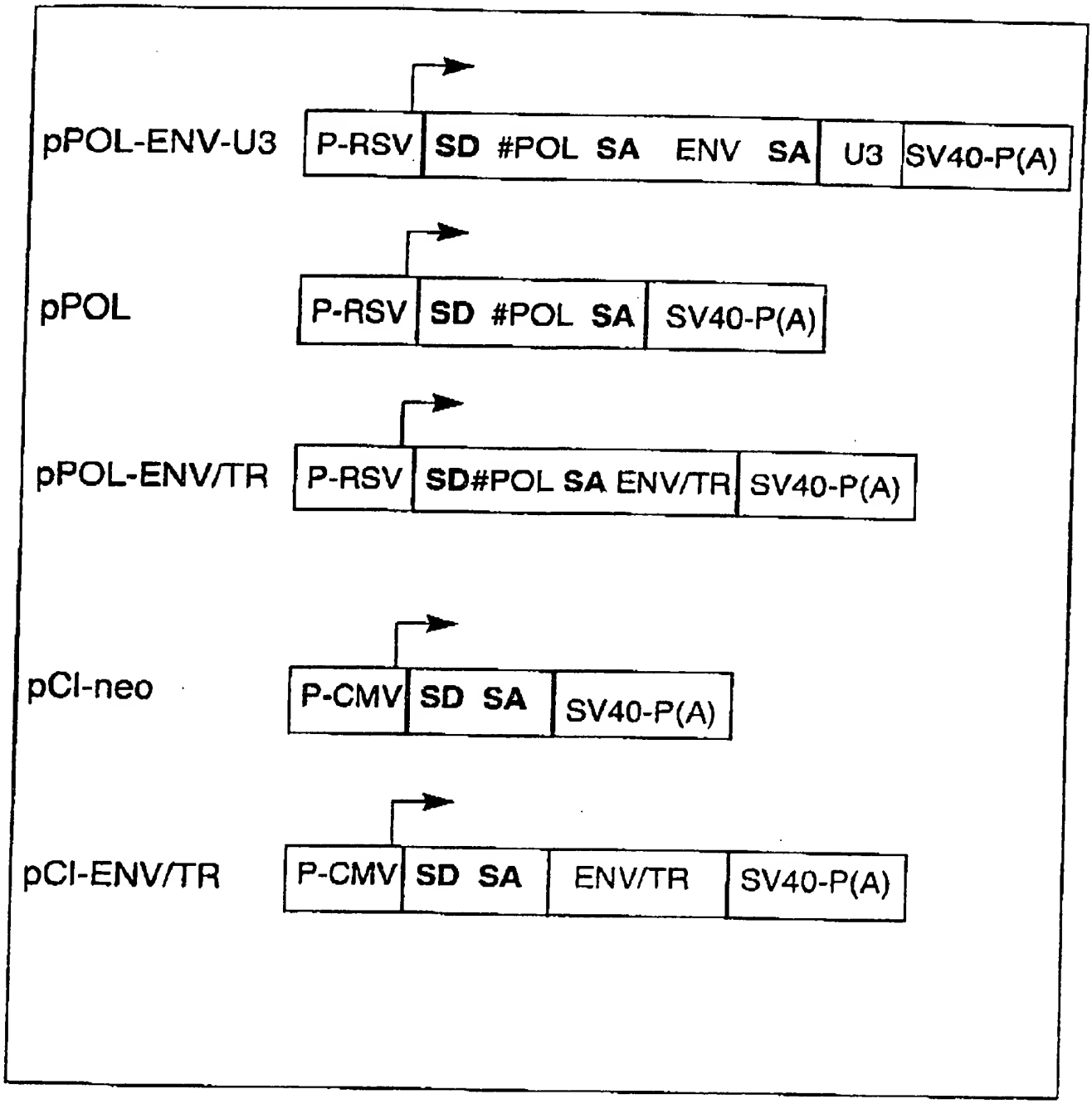


FIGURE 5.

FIGURE 6A.


pPOL-ENV-U3
P-RSV
SD #POL SA ENV SA
U3
SV40-P(A)
pPOL
P-RSV
SD #POL SA
SV40-P(A)
pPOL-ENV/TR
P-RSV
SD#POL SA ENV/TR
SV40-P(A)
pCI-neo
P-CMV
SD SA
SV40-P(A)
pCI-ENV/TR
P-CMV
SD SA
ENV/TR
SV40-P(A)

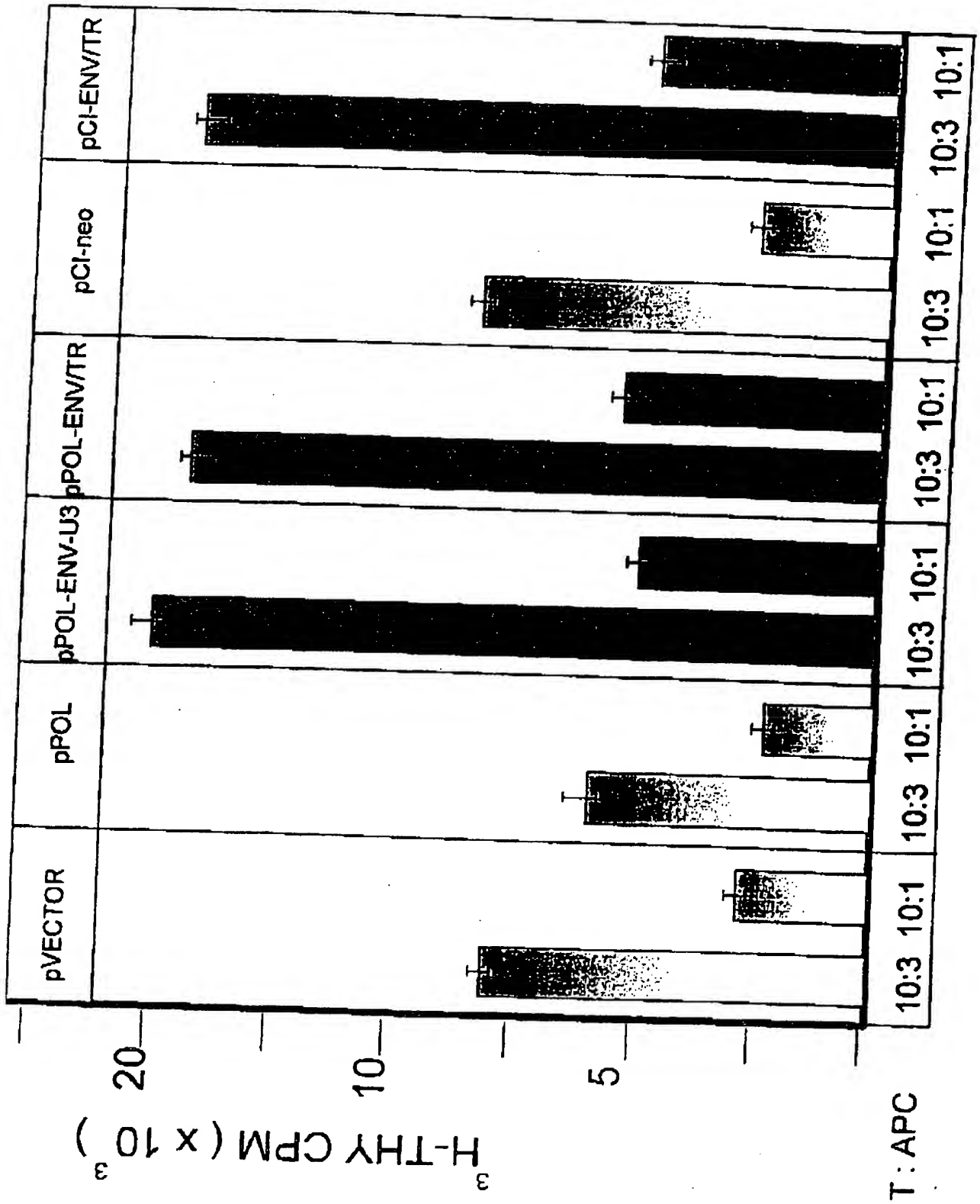

pVECTOR
pPOL
pPOL-ENV-U3
pPOL-ENV/TR
pCI-neo
pCI-ENV/TR
10:3 10:1 10:3 10:1 10:3 10:1 10:3 10:1 10:3 10:1 10:3 10:1
20
10
5
^{3}H-THY CPM (x 10^3)
T : APC

FIGURE 6B.

FIGURE 7A

iddmk1,2 22-5'ltr

CATCTCCCTCAGGAGAAACACCCACGAATGATCAATAAATACTAAGGGGACTCAGAGGCTGGT
GGGATCCTCCATATGCTGAACGTTGGTTCCCGGGGCCCCCTTATTTCTTTCTCTATACTTTGT
CTCTGTGTCTTTTTCTTTTCCAAGTCTTCTTCATTTGCACCTTACGAGAAACATCTCCATCAT
GGTTGTTGGATGGGGGCAA

FIGURE 7B

iddmk1,2 22-3'ltr

CTGCAGGTGTACCCAACAGCTCCGAAGAGACAGTGACATCGAGAACGGGCCATGATGACGATG
GCGGTTTTGTCGAAAAGAAAAGGGGGAAATGTGGGGAAAAGCAAGAGAGATGAGATTGTTACT
GTGTCTGTATAGAAAGAAGTAGACATAGGAGACTCCATTTTGTTCTGTACTAAGAAAAATTCT
TCTGCCTTGAGATGCTGTTAATCTATGACCTTACCCCCAACCCCGTGCTCTCTGAAACATGTG
CCGTGTCAAAcTCAGGGTTAAATGGATTAAGGGTGGTGCAAGATGTGCTTTGTTAAACAGATG
CTTGAAGGCAGCATGCTCATTAAGAGTCATCACCACTCCCTAATCTCAAGTACCCAGGGACAC
AAACACTGCGAAAGGCCGCAGGGACCTCTGCCTAGGAAAGCCAGGTATTGTCCAAGGTTTCTC
CCCATGTGATAGTCTGAAATATGGCCTCGTGGGAAGGGAAAGACCTGACCATCCCCCAGACCA
ACACCCGTAAAGGGTCTGTGCTGAGGAGGATTAGTATAAGAGGAAAGCATGCCTCTTGCAGTT
GAGAGAAGAGGAAGACATCTGTCTCCTGCCCATCCCcTGGGCAATGGAATGTCTCAGTATAAA
ACCCGATTGAACATTCCATCTACTGAGATAGGGAAAAACTGCCTTAGGGCTGGAGGTGGGACA
TGTGGGCAGCAATACTGCTTTGTAAAGCATTGAGATGTTTATGTGTATGTATATCTAAAAGCA
CAGCACTTGATCCTTTACCTTGTCTATGATGCAAACACCTTTGTTCACGTGTTTGTCTGCTGA
CCCTCTCCCCCACTATTGTCTTGTGACCCTGACACATCTCCCTCAGGAGAAACACCCAcgaatg
atcaataaatactaaggggactcagaggctggtgggatcctccatatgctgaacgttggttcc
cggggcccccttatttctttctctatactttgtctctgtgtctttttctttttccaagtcttct
tcatttgcaccttacgagaaacatctccatcatggttgttggatgggggcaa

FIGURE 7C

iddmk1,2 22-env

ATGGTAACACCAGTCACATGGATGGATAATCCTATAGAAGTATATGTTAATGATAGTGTATGG
GTACCTGGCCCCACAGATGATCGCTGCCCTGCCAAACCTGAGGAAGAAGGGATGATGATAAAT
ATTTCCATTGGGTATCATTATCCTCCTATTTGCCTAGGGAGAGCACCAGGATGTTTAATGCCT
GCAGTCCAAAATTGGTTGGTAGAAGTACCTACTGTCAGTCCTAACAGTAGATTCACTTATCAC
ATGGTAAGCGGGATGTCACTCAGGCCACGGGTAAATTATTTACAAGACTTTTCTTATCAAAGA
TCATTAAAATTTAGACCTAAAGGGAAAACTTGCCCCAAGGAAATTCCTAAAGGATCAAAGAAT
ACAGAAGTTTTAGTTTGGGAAGAATGTGTGGCCAATAGTGTGGTGATATTACAAAACAATGAA
TTCGGAACTATTATAGATTAGGCACCTCGAGGTCAATTCTACCACAATTGCTCAGGACAAACT
CAGTCGTGTCCAAGTGCACAAGTGAGTCCAGCTGTCGATAGCGACTTAACAGAAAGTCTAGAC
AAACATAAGCATAAAAAATTACAGTCTTTCTACCTTTGGGAATGGGAAGAAAAAGGAATCTCT
ACCCCAAGACCAAAAATAATAAGTCCTGTTTCTGGTCCTGAACATCCAGAATTGTGGAGGCTT
ACTGTGGCCTCACACCACATTAGAATTTGGTCTGGAAATCAAACTTTAGAAACAAGATATCGT
AAGCCATTTTATACTATCGACCTAAATTCCATTCTAACGGTTCCTTTACAAAGTTGCCTAAAG
CCCCCTTATATGCTAGTTGTAGGAAATATAGTTATTAAACCAGCCTCCCAAACTATAACCTGT
GAAAATTGTAGATTGTTTACTTGCATTGATTCAACTTTTAATTGGCAGCACCGTATTCTGCTG
GTGAGAGCAAGAGAAGGCATGTGGATCCCTGTGTCCACGGACCGACCGTGGGAGGCCTCGCCA
TCCATCCATATTTTGACTGAAATATTAAAAGGCGTTTTAAATAGATCCAAAAGATTCATTTTT
ACTTTAATTGCAGTGATTATGGGATTAATTGCAGTCACAGCTACGGCTGCTGTGGCAGGGGTT
GCATTGCACTCTTCTGTTCAGTCAGTAAACTTTGTTAATTATTGGCAAAAGAATTCTACAAGA
TTGTGGAATTCACAATCTAGTATTGATCAAAAATTGGCAAGTCAAATTAATGATCTTAGACAA
ACTGTCATTTGGATGGGAGACAGGCTTGACTTAGAACATCATTTCCAGTTACAGTGTGACTGG
AATACGTCAGATTTTTGTATTACACCCCAAATTTATAATGAGTCTGAGCATCACTGGGACATG
GTTAGACGCCATCTACAGGGAAGAGAAGATAATCTCACTTTAGACATTTCCAAATTAAAAGAA
CAAATTTTCGAAGCATCAAAAGCCCATTTAAATTTGGTGCCAGGAACTGAGGCAATTGCAGGA
GTTGCTGATGGCCTCGCAAATCTTAACCCTGTCACTTGGATTAAGACCATCAGAAGTACTATG
ATTATAAATCTCATATTAATCGTTGTGTGCCTGTTTTGTCTGTTGTTAGTCTGCAGGTGTACC
CCAACAGCTCCGAAAAAAACAGTGACATCGAGAACGGGCCATGAATGACAAAGGCGGTTTTTG
TTCCAAAAAAAAAAGGGGGAAATTTTGGGGAAAACCAAAAAAATGAAAATGTT

FIGURE 7D

```
ACA TTT GAA GTT CTA CAA TGA ACC CAT CAG AGA TGC AAA GAA AAG CGC CTC CAC GGA 57

GAT GGT AAC ACC AGT CAC ATG GAT GGA TAA TCC TAT AGA AGT ATA TGT TAA TGA TAG 114
  M   V   T   P   V   T   W   M   D   N   P   I   E   V   Y   V   N   D   S 19

TGT ATG GGT ACC TGG CCC CAC AGA TGA TCG CTG CCC TGC CAA ACC TGA GGA AGA AGG 171
  V   W   V   P   G   P   T   D   D   R   C   P   A   K   P   E   E   E   G 38

GAT GAT GAT AAA TAT TTC CAT TGG GTA TCA TTA TCC TCC TAT TTG CCT AGG GAG AGC 228
  M   M   I   N   I   S   I   G   Y   H   Y   P   P   I   C   L   G   R   A 57

ACC AGG ATG TTT AAT GCC TGC AGT CCA AAA TTG GTT GGT AGA AGT ACC TAC TGT CAG 285
  P   G   C   L   N   P   A   V   Q   N   W   L   V   E   V   P   T   V   S 76

TCC TAA CAG TAG ATT CAC TTA TCA CAT GGT AAG CGG GAT GTC ACT CAG GCC ACC GGT 342
  P   N   S   R   F   T   Y   H   M   V   S   G   M   S   L   R   P   R   V 95

AAA TTA TTT ACA AGA CTT TTC TTA TCA AAG ATC ATT AAA ATT TAG ACC TAA AGG GAA 399
  N   Y   L   Q   D   F   S   Y   Q   R   S   L   K   F   R   P   K   G   K 114

AAC TTG CCC CAA GGA AAT TCC TAA AGG ATC AAA GAA TAC AGA AGT TTT AGT TTG GGA 456
  T   C   P   K   E   I   P   K   G   S   K   N   T   E   V   L   V   W   E 133

AGA ATG TGT GGC CAA TAG TGT GGT GAT ATT ACA AAA CAA TGA ATT CGG AAC TAT TAT 513
  E   C   V   A   N   S   V   V   I   L   Q   N   N   E   F   G   T   I   I 152

AGA TTA G 520
  D   *   153
```

FIGURE 7E

k1,2-22-env/fs

ACATTTGAAGTTCTACAATGAACCCATCAGAGATGCAAAGAAAAGCGCCTCCACGGAGATGGTA
ACACCAGTCACATGGATGGATAATCCTATAGAAGTATATGTTAATGATAGTGTATGGGTACCTG
GCCCCACAGATGATCGCTGCCCTGCCAAACCTGAGGAAGAAGGGATGATGATAAATATTTCCAT
TGGGTATCATTATCCTCCTATTTGCCTAGGGAGAGCACCAGGATGTTTAATGCCTGCAGTCCAA
AATTGGTTGGTAGAAGTACCTACTGTCAGTCCTAACAGTAGATTCACTTATCACATGGTAAGCG
GGATGTCACTCAGGCCACGGGTAAATTATTTACAAGACTTTTCTTATCAAAGATCATTAAAATT
TAGACCTAAAGGGAAAACTTGCCCCAAGGAAATTCCTAAAGGATCAAAGAATACAGAAGTTTTA
GTTTGGGAAGAATGTGTGGCCAATAGTGTGGTGATATTACAAAACAATGAATTCGGAACTATTA
TAGATTAGGCACCTCGAGGTCAATTCTACCACAATTGCTCAGGACAAACTCAGTCGTGTCCAAG
TGCACAAGTGAGTCCAGCTGTCGATAG

FIGURE 7F

iddmk1,2 22-ENV

MVTPVTWMDNPIEVYVNDSVWVPGPTDDRCPAKPEEEGMMINISIGYHYPPICLGRA
PGCLMPAVQNWLVEVPTVSPNSRFTYHMVSGMSLRPRVNYLQDFSYQRSLKFRPKG
KTCPKEIPKGSKNTEVLVWEECVANSVVILQNNEFGTIIDZAPRGQFYHNCSGQTQSC
PSAQVSPAVDSDLTESLDKHKHKKLQSFYLWEWEEKGISTPRPKIISPVSGPEHPEL
WRLTVASHHIRIWSGNQTLETRYRKPFYTIDLNSILTVPLQSCLKPPYMLVVGNIVIKP
ASQTITCENCRLFTCIDSTFNWQHRILLVRAREGMWIPVSTDRPWEASPSIHILTEILK
GVLNRSKRFIFTLIAVIMGLIAVTATAAVAGVALHSSVQSVNFVNYWQKNSTRLWNS
QSSIDQKLASQINDLRQTVIWMGDRLDLEHHFQLQCDWNTSDFCITPQIYNESEHH
WDMVRRHLQGREDNLTLDISKLKEQIFEASKAHLNLVPGTEAIAGVADGLANLNPVT
WIKTIRSTMIINLILIVVCLFCLLLVCRCTPTAPKKTVTSRTGHE

```
                                                                    63
ACATTTGAAGTTCTACAATGAACCCATCAGAGATGCAAAGAAAAGCGCCTCCACGGAGATGGT

                                                                     2
                                                                   M  V
                                                                   126
AACACCAGTCACATGGATGGATAATCCTATAGAAGTATATGTTAATGATAGTGTATGGGTACC
                                                                    23
 T  P  V  T  W  M  D  N  P  I  E  V  Y  V  N  D  S  V  W  V  P
                                                                   189
TGGCCCCACAGATGATCGCTGCCCTGCCAAACCTGAGGAAGAAGGATGATGATAAATATTTC
                                                                    44
  G  P  T  D  D  R  C  P  A  K  P  E  E  E  G  M  M  I  N  I  S
                                                                   252
CATTGGGTATCATTATCCTCCTATTTGCCTAGGGAGAGCACCAGGATGTTTAATGCCTGCAGT
                                                                    65
  I  G  Y  H  Y  P  P  I  C  L  G  R  A  P  G  C  L  M  P  A  V
                                                                   315
CCAAAATTGGTTGGTAGAAGTACCTACTGTCAGTCCTAACAGTAGATTCACTTATCACATGGT
                                                                    86
  Q  N  W  L  V  E  V  P  T  V  S  P  N  S  R  F  T  Y  H  M  V
                                                                   378
AAGCGGGATGTCACTCAGGCCACGGGTAAATTATTTACAAGACTTTTCTTATCAAAGATCATT
                                                                   107
  S  G  M  S  L  R  P  R  V  N  Y  L  Q  D  F  S  Y  Q  R  S  L
                                                                   431
AAAATTTAGACCTAAAGGGAAAACTTGCCCCAAGGAAATTCCTAAAGGATCAAAGAATACAGA
                                                                   128
  K  F  R  P  K  G  K  T  C  P  K  E  I  P  K  G  S  K  N  T  E
                                                                   504
AGTTTTAGTTTGGGAAGAATGTGTGGCCAATAGTGTGGTGATATTACAAAACAATGAATTCGG
                                                                   149
 V  L  V  W  E  E  C  V  A  N  S  V  V  I  L  Q  N  N  E  F  G
                                                                   567
AACTATTATAGATTTAGGCACCTCGAGGTCAATTCTACCACAATTCCTCAGGACAAACTCAGT
                                                                   170
  T  I  I  D  L  G  T  S  R  S  I  L  P  Q  L  L  R  T  N  S  V
                                                                   601
CGTGTCCAAGTGCACAAGTGAGTCCAGCTGTCGATAG
                                                                   181
  V  S  K  C  T  S  E  S  S  C  R  *
```

FIGURE 7G

FIGURE 7H

iddmk1,2 22-POL

FTIPLAEQDCEKFAFTIPAINNKEPATRFQWKVLPQGMLNSPTICQTFVGRALQPVRDKFSDC
YIIHYFDDILCAAETKDKLIDCYTFLPAEVANAGLAIASDKIQTSTPFHYLGMQIENRKIKPQ
KIEIRKDTLKTLNDFQKLLGDINWIRPTLGIPTYAMSNLFSILRGDSDLNSKRMLT

FIGURE 8A

k1,2-1

gtaaatgacacctatgatgcactgccacccttcactgtttcaccctgaacatctgctttttac
atctaagtgattgtacccaataaatagtgtggagaccagagctctgagccttttgcagcctcca
ttttgcaactggtcccctggctcccacctttatgaactcttaacctgtcttttctcattcctttt
gtcaccattggactttgggtaccctacgggtggtgttgaggctgtcaccgcacattaa

FIGURE 8B

k1,2-10

gtttagttaatctataatctatagagacaatgcttatcactggcttgctgtcaataaatatgtg
ggtaaatctctgttcaagactctcagctttgaagctgtgagacccctgatttcccactccacac
ctctatatttctgtgtgtgtgtctttaattcctccagtgttgctgggttagggtctcctcgacg
agctgtcgtgc

FIGURE 8C

k1,2-16

aactcagctgctgcacagtggtcgagcctccagagctcatgccattgcagtggtcagagcctg
gccctcctcttcctgcatagaacctggattcaatctgtaaggtgggaagtgcagcagcagaga
actctggccttgcagagagtccctgttcccacttcactttcctttcaccaaataaaaccctg
ctttcactcatgcatcaaattgtctgtgagcctacatttttgtggccatgggacaagaacacc
atctttagctgagctagggaaaagtcctgca

FIGURE 8D

k1,2-17

gatgtgaccactgtgacctacctacactggagatggctcacacttccttacccttcccctgct
gtaccaataaataacagcacagcctgacattcggagccattaccggtctttgtgacttggtgg
tagtggtatcccctagggcccagctgtcttttcttttatctctttgtcttgtgtctttatttc
tatgagtctctcgtctccgcacatggggagaaaaacccatagaccctgtagggctg

FIGURE 8E

k1,2-26

ctcacaaaaataataaaagcttctgttggccattcttcagatcttcatctcttgtgaggatcc
ccctgtacatgtaaaaatgtaataaaacttgtatcctttctcctcttaatctgtcttgcatca
atatcattcctagacccagtcagagatgggtggaggtgagccgtacatttcccta

FIGURE 8F

k1,2-27

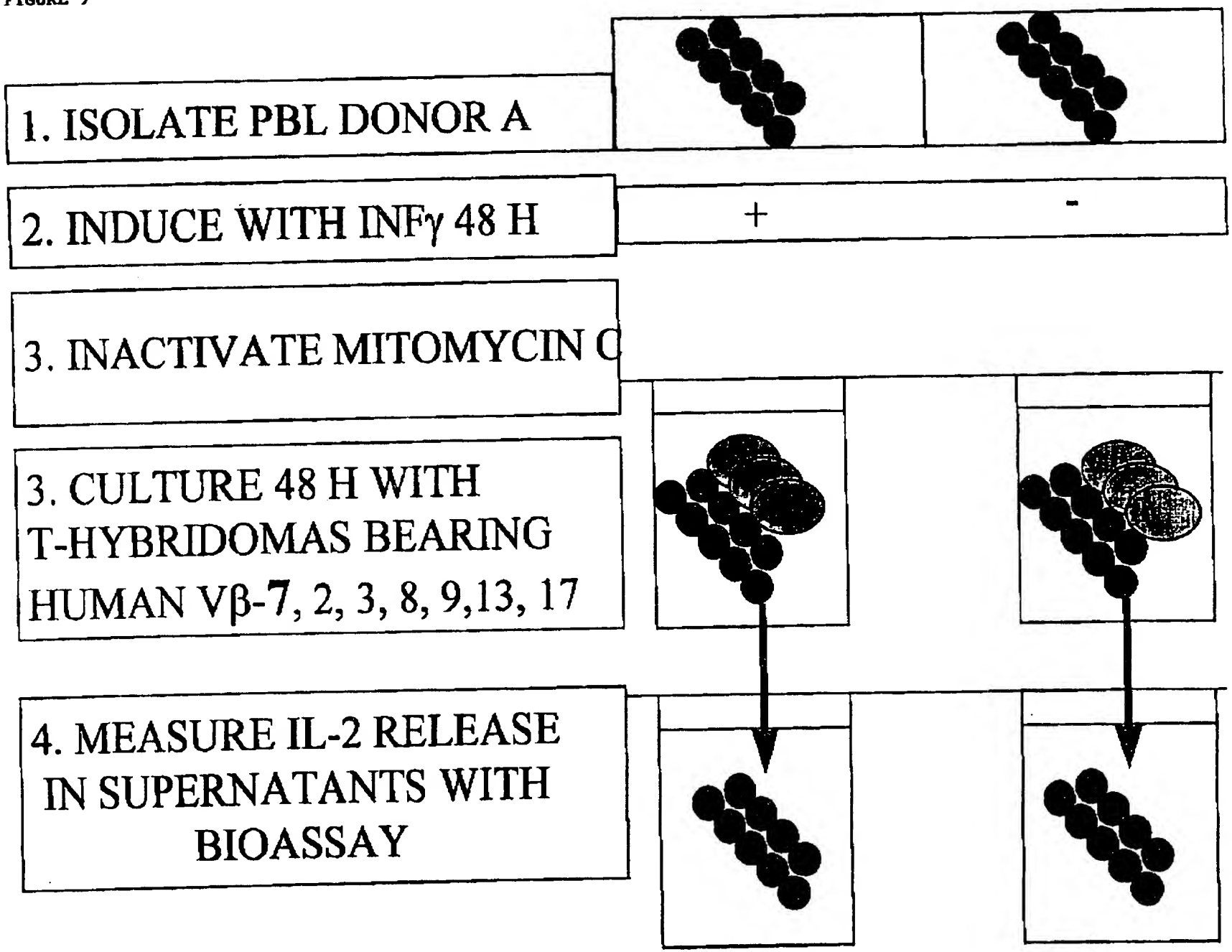
cagagaactccagccagctgtgatggagcctcaggaagttcacagttgcagcaggaaggagcctggc
tgctcctcttcctgtgtggaacctgggattagaacaggctggcaggaagtgctttagcagggactct
ggcctactcacactccttgtttccccccttttcttcctttcactcaataaagccctgtcttactcac
cattcaaattgtctgtgagcctgaattttcatggctgtgggacaaagaaccctatttttagctgaac
taaggaaaattcctgcaaa

FIGURE 8G

k1,2-4

gtgattgtctgctgaccctctccccacaattgtcttgtgaccctgacacatcccccctcttcga
gaaacacccgcggatgatcaataaatattaagggaactcagaggctggcaggatcctccatat
gctgaacgctggttgccccgggtcccctttctttctttctctatactttgtctctgtgtctttt
tcttttccaaatctctcgtcccaccttacgagaaacacccacaggtgtgtccgggcaacccaa
cgccacataaca

FIGURE 9


1. ISOLATE PBL DONOR A
2. INDUCE WITH INFγ 48 H
+
-
3. INACTIVATE MITOMYCIN C
3. CULTURE 48 H WITH T-HYBRIDOMAS BEARING HUMAN Vβ-7, 2, 3, 8, 9,13, 17
4. MEASURE IL-2 RELEASE IN SUPERNATANTS WITH BIOASSAY